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EFFECT OF HIGH FAT DIETS CONTAINING NO CHOLESTEROL ON THE PROPERTIES OF RABBIT SERUM LIPOPROTEINS AND THE CATABOLISM OF VERY LOW DENSITY LIPOPROTEINS

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ABBREVIATIONS

LPL	Lipoprotein lipase
PHP LPL	Post-heparin plasma lipoprotein lipase from intact animals
EHP LPL	Extra-hepatic post-heparin plasma lipoprotein lipase from supradiaphragmatic animals
LCAT	Lecithin-cholesterol acyl transferase
VLDL	Very low density lipoprotein ($d < 1.006$ g/ml)
IDL (LDL_1)	Intermediate density lipoprotein ($1.006 < d < 1.019$ g/ml)
LDL (LDL_2)	Low density lipoprotein ($1.019 < d < 1.063$ g/ml)
HDL	High density lipoprotein
apo A-I	Apolipoprotein A-I
apo A-II	Apolipoprotein A-II
apo B	Apolipoprotein B
apo C-I	Apolipoprotein C-I
apo C-II	Apolipoprotein C-II
apo E	Apolipoprotein E
FFA	Free fatty acid
(HMG)-CoA reductase	3-hydroxy-3-methylglutarate-CoA reductase
SDS	Sodium dodecyl sulfate

I. LITERATURE REVIEW

A. Serum Cholesterol, Lipoproteins and Atherogenesis

Historically, most workers in the atherosclerosis area see cholesterol as a focal element in the etiology of cardiovascular disease. In 1910, the presence of cholesterol in the lesions of diseased arteries was described (1). Since then many studies have confirmed that both free and esterified cholesterol accumulate in the aorta, coronary arteries, cerebral vessels, and other large arteries at different rates in different persons or in different populations. Epidemiological studies have repeatedly shown high degrees of correlation between the intake of cholesterol and other lipids with the prevalence of coronary heart disease in various populations (2). Animal studies show that experimentally-induced hypercholesterolemia is followed by the accumulation of cholesterol in the large arteries, and that under certain conditions even more advanced manifestations of atherosclerosis are produced (3). Finally, recent work on cultured cells shows that certain lipoproteins from normal or hypercholesterolemic serum are rapidly taken up by arterial smooth muscle and endothelial cells (4, 5) and by skin fibroblasts (6).

The LDL is the primary carrier of cholesterol in the plasma of persons with plasma cholesterol concentrations higher than 150 mg/dl. The apoprotein of LDL, apo-B, is found in the arterial lesion (7, 8). Familial hyperbetalipoproteinemia, in which high levels of LDL are present, is associated with premature arteriosclerosis and coronary heart disease. The LDL fraction is usually elevated in experimental animals that eat atherogenic diets (6).

The role of very low density lipoproteins (VLDL) in atherogenesis is not as well established. Some epidemiological studies, however, have shown significant correlations between hypertriglyceridemia and coronary heart disease (9). The apoproteins characteristic of VLDL (apo-C and apo-B) have been found in human arterial lesions (7, 10). Plasma VLDL is the precursor of plasma LDL (11, 12) and it may be atherogenic.

The idea that chylomicrons could be atherogenic has been proposed by Zilversmit. This theory is based on the hypothesis that the interaction of triglyceride-rich lipoproteins with arterial lipoprotein lipase is an atherogenic process (13). The process is assumed to involve the binding of chylomicrons to the arterial surface, the hydrolysis of triglyceride by arterial lipoprotein lipase and the subsequent internalization of cholesterol-enriched chylomicron remnants by the arterial smooth muscle cells. It is the chylomicron remnant, then, which is thought to be atherogenic.

B. Lipoprotein Metabolism

1. Interconversions of Lipoproteins

Lipoprotein particles are the major transport vehicles of fat soluble materials in the circulation. All lipoproteins are discrete particles with finite dimensions and compositions and are frequently characterized as such. However, it should be remembered that all plasma lipoproteins are metabolically related and that the interaction of single particles with one another is perhaps the best physiological approach in understanding lipoprotein metabolism.

The various plasma lipoproteins are probably derived from the liver and the intestine. Both of these tissues normally produce triglyceride-rich

lipoproteins. The liver secretes VLDL in response to a dietary load of either carbohydrate or fatty acids, while the intestine responds to the presence of dietary fat by secreting chylomicrons. These lipoproteins are rich in triglyceride but also contain free and esterified cholesterol, phospholipids and various apoproteins. One apoprotein (apo B protein, apo-LDL) appears to be an integral part of the lipoprotein, while the other proteins, and lipids as well, are subject to exchange or transfer to other lipoprotein complexes (14, 15). Free cholesterol readily exchanges between all lipoproteins and between lipoprotein and cell membranes as well (16). The cholesteryl ester may require a plasma protein to effect exchange (17, 18) or transfer (19). Phospholipids exchange via a plasma protein fraction as well (20). The apolipoproteins (apo A-I, apo E or arginine-rich, and apo C) seem to move freely from one lipoprotein to another (14, 22). Some of these apolipoproteins are co-factors in the degradation of triglyceride-rich lipoproteins and then appear to be delivered to the higher density fractions (23) and are used again via transfer to a newly secreted VLDL or chylomicron (14).

A precursor-product relationship has been known to exist between VLDL and LDL for some time (24). In normal individuals the interaction of VLDL with LPL seems to give rise to an intermediate density fraction (IDL or LDL_1) which is further degraded by a less well-defined pathway possibly involving lecithin-cholesterol acyl transferase (LCAT). In normal individuals, the flux of apo B through these three fractions is approximately equal, indicating the existence of a possible quantitative conversion. In hypertriglyceridemic subjects, the apo B flux in VLDL exceeds that in the LDL fraction, suggesting that a part of the VLDL or IDL may be removed by another pathway (25).

When VLDL proteins are radiolabeled, a transient appearance of the label has been found in a lipoprotein of intermediate density between VLDL and LDL (IDL, LDL, $1.006 < d < 1.019$ g/ml) (26). Thus it appears that the intermediate particle formed during the catabolism of triglyceride-rich lipoproteins exists as a free lipoprotein complex in plasma. Since LPL contains only a single active site per molecule (27), and since many molecules of triglyceride are contained in the triglyceride-rich lipoproteins, these intermediates are expected to occur. The triglyceride-depleted particles formed in this fashion are called remnant lipoproteins.

Various techniques have been used to investigate the character of these remnant particles. All have involved the isolation of the peripheral LPL system from the hepatic one allowing the accumulation of the remnant fraction. The functionally hepatectomized rat has been used to produce large amounts of remnant lipoproteins (20, 28), but this technique is limited by the presence of endogenous triglyceride-rich lipoproteins which may contaminate this system. A second method involves the use of whole organ perfusions, especially heart and adipose tissues (29, 30, 31). Although these procedures allow the use of well-defined systems, the amount of remnant lipoproteins produced is small. Another approach has involved using either purified LPL (32) or whole post-heparin plasma (15) with the *in vitro* incubation of isolated chylomicrons or VLDL. While large amounts of remnants can be produced in this way, in the case of whole post-heparin plasma, triglyceride hydrolysis is due to a mixture of lipases including the hepatic lipase which appears to play no direct role in remnant formation. Lastly, a combination of functionally hepatectomized animals and *in vitro* post-heparin plasma-lipoprotein incubations may serve to eliminate the

hepatic-lipase activity and allow the preparation of remnant lipoprotein in sufficient quantities to characterize. This technique, used in the present studies, may also shed further light on the role of the hepatic lipase in remnant formation by comparing post-heparin plasma lipase remnant production between intact and functionally hepatectomized animals.

Since apo C lipoproteins are known to recycle between triglyceride-rich lipoproteins and HDL particles, remnants would be expected to become depleted of the C peptides. This depletion has been shown to occur in the rat (20). The apo B and apo E proteins appear to be retained. The cholesteryl ester component has not been demonstrated to be a substrate for lipolysis by lipase enzymes but phospholipids (i.e. phosphatidyl choline and phosphatidyl ethanolamine) may be attacked by a phospholipase present in the LPL system (33, 34). As a result, remnant phospholipids become relatively enriched in sphingomyelin. The rates of this phospholipase activity are not high when compared with the lipase activities. Yet they may be significant when considered in terms of surface-volume relationships of the lipoproteins since the phospholipid makes up most of the surface of the VLDL and chylomicron while the triglycerides make up a large portion of the core of these particles (35). This phospholipase activity, then, may play an important role in maintaining the spherical nature of the triglyceride-rich particle during triglyceride hydrolysis. A loss of VLDL lecithin has been shown to occur (36) in the whole post-heparin plasma case, but it should be remembered that post-heparin plasma contains the hepatic lipase which has distinct phospholipase activity (37). Furthermore, phospholipid is able to freely exchange between VLDL and HDL during triglyceride hydrolysis <u>in vivo</u> (38). Thus, some phospholipid may be lost in this

fashion as well. Whatever mechanisms are involved, remnants are significantly depleted of triglyceride, phospholipid and protein and retain most of their cholesterol and cholesteryl esters.

2. Chylomicron Clearance

The sequence of events in the conversion of VLDL to LDL and the possible removal of excess IDL from plasma appears to have a parallel in chylomicron metabolism. The chylomicrons formed in the intestinal mucosal cells in response to dietary fat are carried via the lymphatic system to the thoracic duct. These particles are subsequently mixed with the components of the circulatory system at the subclavian vein. The nature of these particles depends on both the nature and the availability of the lipids in the intestinal lumen (39, 40) and the protein synthesizing capabilities of the intestinal mucosal cells (41, 42, 43). Zilversmit et al. (44) has summarized the relationship of dietary fat to chylomicron size distribution on the basis of the relative rates of triglyceride transport per mucosal cell and the synthetic rates of the surface material. Their conclusions were that lymph chylomicrons are larger, (1) during the absorption of a high fat meal than during a low fat meal, (2) during peak fat absorption periods, (3) during the absorption of unsaturated fats than during saturated fat absorption, and (42) when protein synthesis is inhibited.

After the secretion of the chylomicrons from the gut, they contain the apolipoproteins, apo A-I, apo A-II, apo B, and possibly others. Then upon their entry into the circulation, they acquire apoproteins C and E, and are degraded by lipoprotein lipase activity in conjunction with apo C-II (45). During this process, the chylomicron loses its major component,

triglyceride. Concomittant with this removal, the particle also loses surface components such as apo A-I, apo A-II, and phospholipids. The triglyceride-depleted particle which remains is also referred to as a remnant. These remnants containing high levels of cholesteryl esters and apo B protein are thought to be taken up by the liver (46, 47, 48). In this way, either dietary cholesterol or intestinally-synthesized cholesterol can enter the liver and take part in the regulation of endogenous cholesterol synthesis.

3. Enzymes of Lipid Metabolism in Plasma

a. Triacylglycerol Hydrolases

The intravenous injection of heparin causes the rapid release of lipolytic activity into plasma which is active against triglyceride-rich lipoproteins or certain long-chain triglyceride emulsions to which specific apolipoproteins (apo C-II) have been added. This enzyme activity has been termed "clearing factor lipase", or "lipoprotein lipase" (LPL) (EC 3.1.1.3) (49). Post-heparin plasma is now known to contain at least two distinct types of hydrolases for long-chain triglycerides, one or more "peripheral" lipoprotein lipases which are primarily active against native chylomicrons, VLDL, and apo C-II activated triglyceride emulsions and an hepatic lipase which is more active against lipid emulsions in the absence of apo C-II.

In comparison to hepatic lipase, the peripheral LPL is inhibited by polycations such as protamine and by molar sodium chloride. Additionally, the physical and chemical nature of the substrate is important in differentiating the two activites (50, 51). The peripheral enzyme is activated by apo C-II (52) and it is inhibited by protamine sulfate, which has provided

an empirical method for quantifying the two activites in post-heparin plasma from rats (53) and man (54).

The hepatic triglyceride hydrolase is enzymologically distinct from peripheral LPL (55, 56). The hepatic enzyme attacks some artificial substrates which are poor substrates for the peripheral LPL. VLDL and chylomicrons, which are readily degraded by the peripheral LPL, are poor substrates for the hepatic lipase (55). Protamine and molar sodium chloride do not inhibit the hepatic enzyme as they do the peripheral lipase and no co-factor requirement has been demonstrated for the hepatic lipase. In addition, some evidence indicates that the hepatic enzyme is responsible for the hydrolysis of the 2-monoglyceride residue which is the major product of peripheral LPL against the triglyceride substrate (50).

Triglyceride hydrolases from other tissues studied are enzymologically similar to the peripheral LPL (57, 58), though the number of distinct molecular species remains uncertain. In adipose tissue, two forms of peripheral LPL with different apparent molecular weights have been extracted (59). The precise enzymological determination of the relative contribution of hepatic and peripheral LPL activities in post-heparin plasma is difficult, especially since the activities of the two enzymes are affected by the physical nature of the substrate employed (50, 51). Yet the method devised by Krauss et al. (53) to selectively measure these two types of activity has been used with some utility in the rat (53), dog (60), and man (54), and does provide a reasonable estimate of the contributions of each enzyme to the total post-heparin plasma activity.

b. The Cholesterol Acylating System

The enzyme, lecithin-cholesterol acyl transferase (EC 2.3.1.43) (LCAT), is the major source of plasma cholesteryl ester production in man. This enzyme catalyzes the transfer of the C-2 fatty acyl chain from lecithin to cholesterol yielding the cholesteryl ester (61). In addition to maintaining normal plasma lipoprotein composition and structure, LCAT may have a role in cholesterol transport. LCAT, by reducing the content of free cholesterol on the surface of its natural substrate, HDL, allows additional free cholesterol to be transferred from the membrance of peripheral tissues to the HDL. The esterified cholesterol produced in this way can then be transported to the liver and further catabolized (63).

In addition, the catabolism of the triglyceride-rich lipoproteins may require both lipoprotein lipase (LPL) and LCAT. As LPL reduces the size of the neutral lipid core of either chylomicrons or VLDL, an excess of surface components may be created (i.e. phospholipid and free cholesterol). LCAT may act in concert with LPL to then reduce the excess phospholipid and cholesterol on the surface of these particles, since the neutral cholesteryl ester would be transferred to the interior of the lipoprotein in question (64). Another possibility is that as the triglycerides are removed, their core volume is reduced and the excess surface components form bilayer lipid folds. These folds form disc-like particles or vesicles on which LCAT can act to make spherical HDL.

In the rabbit, however, it appears that plasma LCAT activity is less significant. Rose (62) has concluded that LCAT is probably not responsible for the increase in cholesteryl esters in the blood of cholesterol-fed

rabbits. These esters appear to be synthesized at sites other than the circulation, presumably the liver and intestinal tract.

C. The Hepatic Metabolism of Free Fatty Acids

The free fatty acid (FFA) available to the liver in a normal animal can be derived from exogenous or endogenous sources. In the fasting or fat-free fed state, the major source of FFA is probably adipose tissue triglycerides or carbohydrates. In the fed state, especially in high fat diets, the primary source of plasma FFA is dietary triglycerides (47). When animals or man are fed a high fat diet of a particular fatty acid composition, the plasma FFA and plasma and hepatic triglyceride become enriched in the fed fatty acid within a short time. Since the triglyceride fatty acids of adipose tissue appear to turn over at a slower rate than those of liver or plasma (48, 65), the composition of the plasma FFA therefore reflects the composition of ingested triglyceride.

The FFA taken up by the liver is primarily esterified to triglyceride. To a lesser extent it may be incorporated into phospholipid or cholesteryl esters. Depending upon the metabolic needs of the animal, it may also be oxidized to CO_2 and ketone bodies. The triglycerides formed will then be either stored in the liver or secreted into the plasma as the major constituent of the VLDL in conjunction with other lipid and protein components.

D. The Regulation of Hepatic Cholesterogenesis by Free Fatty Acids

As triglycerides are secreted by the liver in the VLDL fraction, phospholipids, cholesterol and proteins are also secreted in certain specific proportions. These other lipids are important in stabilizing the spherical nature of the VLDL particle and to allow the hydrophobic triglyceride to

exist in the aqueous plasma compartment. The specific molar ratios of phospholipid and cholesterol related to triglyceride have been shown to be altered by various factors. The structure and quantity of FFA (66), the rate of triglyceride output by the liver (66), the sex of the animal (67), and fat feeding (68) all have been shown to alter this ratio. Further, when oleic acid was added to the perfusate of isolated rat livers, the incorporation of $^{3}H_2O$ in cholesterol was stimulated. Additionally (69), the activity of hepatic 3-hydroxy-3 methylglutarate (HMG)-CoA reductase (EC 1.1.1.34), the rate limiting step in cholesterol biosynthesis (70), was also stimulated. When exogenous cholesterol was added to the medium along with the oleate, HMG-CoA reductase activity was depressed while the output of triglyceride continued with little change (71). Thus dietary cholesterol appears to inhibit or regulate hepatic cholesterogenesis.

When other fatty acids were used in the medium, the output of cholesterol was in the order 18:1 > 18:2 = 16:0 and the output of triglyceride was in the order 18:1 = 18:2 > 16:0. This work (72) has suggested that the molar ratio of cholesterol to triglyceride in the secreted particle was greater when palmitic acid (16:0) was the substrate than when either oleic or linoleic acids were infused. In summary then, it appears that not only do plasma FFA stimulate cholesterogenesis, but the specific type of FFA have varying effects on this stimulation as well.

E. Characteristics of Cholesterol-Induced Hyperlipoproteinemia in Animals and Man: Current Theories

Five major changes have been consistently observed after cholesterol feeding (60). These changes are: 1) the occurrence of cholesterol-enriched VLDL (B-VLDL); 2) an increase in IDL (d = 1.006-1.019 g/ml); 3) an increase

in LDL; 4) the occurrence of HDL_c; and 5) a decrease in typical HDL. The B-VLDL have been defined as β-migrating lipoproteins which float at d < 1.006 g/ml by preparative ultracentrifugation. The apoproteins of these cholesterol-rich B-VLDL undergo changes as well, being relatively high in the arginine-rich lipoprotein (73, 74), and are comparable to the situation seen in human patients with type III hyperlipoproteinemia (73, 75, 76). In this disease, there are abnormally high levels of LDL_2 (1.019 < d < 1.063 g/ml) and triglyceride-poor cholesteryl ester-enriched VLDL.

The HDL_c seen in cholesterol-fed animals has characteristics in common with both LDL and the typical HDL. As the plasma cholesterol level increases, HDL_c become richer in cholesteryl esters and float at lower densities. The HDL_c particles are similar to LDL with respect to composition and size, but are quite different with respect to apoprotein content, since HDL_c lack the B apoprotein (apo LDL) and primarily contain the arginine-rich protein (apo E) and apo A-I.

The origin of HDL_c is undetermined. It may be a remnant of intestinal lipoproteins or it may be due to an overloading of typical HDL with cholesterol (60).

A number of possible origins for the cholesterol-induced B-VLDL have been suggested. They may be directly synthesized by intestinal mucosa cells in response to cholesterol feeding. Yet evidence from the analysis of thoracic duct lymph from the cholesterol-fed dog indicates that only large triglyceride-rich particles are present in the d < 1.006 g/ml fraction (60). A second possible explanation is that the B-VLDL and IDL are remnants of cholesterol induced intestinal or liver lipoproteins. This theory is supported by the work of Zilversmit et al. (21). Their data in the cholesterol-fed rabbit indicates

that nearly two-thirds of the esterified cholesterol in the d < 1.006 g/ml fraction is derived from chylomicrons. They therefore concluded that the metabolic origin of hypercholesterolemic VLDL cholesteryl esters is derived from dietary sources, which has helped substantiate the chylomicron remnant hypothesis of atherogenesis. Additionally, there appears to be a defect in the removal of chylomicron remnants from the plasma of hypercholesterolemic rabbits (21). Therefore, the B-VLDL and IDL of cholesterol-fed animals may represent products of catabolism due to the action of triglyceride lipases on lipoproteins of intestinal origin.

Furthermore, Mahley et al. studied a large number of lipoprotein changes in dogs treated with cholesterol, bile acids and a thyroid inhibitor (79). These authors also have suggested that in the hyperresponders the B-VLDL are probably chylomicron remnants which have accumulated due to a defective catabolism of the lipoprotein synthesized to carry the excess dietary cholesterol. Their conclusion, however, does not necessarily state that the B-VLDL are chylomicron remnants. Changes in triglyceride lipase activities accompanying cholesterol feeding in these same dogs have also been measured (66). The most significant change in lipolytic activity in the experiments is a reduction in the hepatic lipase activity in the case of the cholesterol-induced hyperlipoproteinemia. Whether these preliminary findings suggest a catabolic defect in the hyperresponders that may explain the differences between the two groups of dogs remains to be determined.

Contrary to the remnant hypothesis of Zilversmit, Shore et al. have proposed an explanation for the origin of B-VLDL in type III and hypothyroid subjects and in cholesterolemic rabbits as well (78). In these conditions

LDL_2 (probably a product of VLDL catabolism) has relatively more of the arginine-rich protein than usual. The LDL_2, however, often contain very little of the arginine-rich protein and relatively more of the other VLDL protein (e.g. apo B and apo C peptides). If the arginine-rich protein is present in relatively small amounts in the serum VLDL, it is likely to be present only in very small amounts in the LDL_2, even if the LDL_2 are elevated (74). The rabbits fed excess cholesterol appear to synthesize considerable quantities of triglyceride-poor VLDL that are very rich in this protein. In addition, these particles are as large or larger than the triglyceride-rich VLDL--not smaller as would be expected if they were "remnants" of the triglyceride-rich particle. Consequently these authors have concluded that the arginine-rich B-VLDL may be derived from a unique VLDL species, enriched in the arginine-rich protein and probably derived by synthetic processes in the liver.

F. The Effect of Cholesterol-Free, High-Fat, Semi-Synthetic Diets in the Rabbit Atherosclerosis Model

In contrast to diets containing supplementary cholesterol, hypercholesterolemia has also been described in rabbits fed certain semi-synthetic diets devoid of exogenous cholesterol (80-83) and containing saturated fat. While the atherogenicity of this type of diet has been studied in some depth (84-87), the effect of it on the serum lipoproteins of rabbits has not previously been reported. The present studies, therefore, were undertaken in order to see what effect semi-synthetic diets, containing no cholesterol and high in either saturated or unsaturated fat would have on the serum lipid and lipoprotein concentrations in the rabbit. Also, whether similar lipoprotein alterations analogous to those seen in cholesterol-induced hyperlipoproteinemia exist were to be investigated.

Lastly, in order to shed further light on the subject of normal and altered VLDL catabolism, experiments utilizing both hepatic and peripheral triglyceride lipase preparations and the *in vitro* production of VLDL remnants were accomplished. The effect of the diets on these parameters was also studied.

II. MATERIALS AND METHODS

A. Preparation of Egg Yolk Phosphatidylcholine

Egg phosphatidylcholine was prepared according to the method of Singleton *et al.* (88). One liter of acetone was added to 500 g of fresh egg yolk, and the mixture was blended in a Sorvall Omni-Mixer (Model 17105, Instrument Products Division, Sorvall Operations, Newtown, Conn.) at 25°C. The mixture was allowed to stand for 30 minutes, filtered and the acetone extract was discarded. The remaining solids were washed 3 times with 200 ml of cold acetone, and then suspended in 1 liter of 95% ethanol. After standing 1 hour, the mixture was filtered and the extraction was repeated with 500 ml of 95% ethanol. The combined extracts were dried under vacuum, and the crude phospholipids were redissolved in 2 portions of 300 ml each of hexane. The 600 ml extract was concentrated to 200 ml and then rapidly poured into 1 liter of cold acetone and allowed to stand until the supernatant cleared. After decanting the acetone phase, the precipitated phospholipids were washed with cold acetone. This acetone-hexane step was repeated and the phospholipids were dried and redissolved in chloroform to give a 10% solution. The phospholipid was stored under nitrogen gas at -20°C until purification via column chromatography was performed.

Purification of the phospholipid was carried out using silicic acid columns essentially according to the method of Bingham et al. (89).

Silicic acid and Hyflo Supercell (2:1) were mixed and washed twice with chloroform-methanol (2:1). Residual solvent was removed under vacuum and the material was dried in a 120°C oven for several hours. The silicic acid-Supercell was then dispersed in chloroform-methanol (2:1) and packed in a column 2.5 cm x 44 cm with glass wool at the bottom. Lecithin (2.4 g, 100 g/ml) was applied to the column at a flow rate of 0.7 ml/minute. Elution of the lecithin was carried out with chloroform-methanol (2:1) with periodic monitoring of the fractionation by thin-layer chromatography ($CHCl_3$:MeOH:H_2O:HCOOH, 65:25:4:4). The first 175 ml of eluted solvent containing mainly neutral lipids and some phosphatidylcholine was collected. The phosphatidylcholine was dried under vacuum and dissolved in chloroform in a final concentration of 40 mg/ml.

B. Buffer System

Experiments were performed in sodium veronal buffer, pH 7.4, in saline containing 0.025% EDTA, unless otherwise specified.

C. Separation of Lipoproteins from Rabbit Plasma

Lipoproteins were prepared as described by Lindgren et al. (90) and Meusing and Nishida (91).

Each rabbit was bled via a marginal ear vein and the whole blood was collected in citrated centrifuge tubes. The plasma was separated from the whole blood and EDTA was added to a final concentration of 0.025%. Sodium azide was added at a level of 2.0 mM to prevent bacterial growth. The plasma was then centrifuged at 8000 X g for 10 minutes in a SS-35 rotor using a

Sorval RC-5 refrigerated centrifuge. Chylomicrons, when present, were removed from the top of the centrifuge tubes by aspiration. The plasma was then centrifuged for 24 hours at 37,000 rpm (100,000 x g) in a Ti 50 rotor with a Model L3-50 or L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The top VLDL layer was removed by aspiration. A 1-hour centrifugation at 37,000 rpm was carried out to remove any remaining chylomicrons. The VLDL was further purified by repeating the 24-hour centrifugation. After removing the VLDL, the remaining plasma components were collected through a hole punctured in the bottom of each tube and the fractions from pairs of rabbits were pooled. The density of these fractions was increased by the addition of solid sodium bromide to 1.019 g/ml and were again centrifuged in a Ti 50 rotor for 48 hours at 37,000 rpm to float the LDL_1 (IDL) fraction. The IDL layer was removed by aspiration from the tops of the tubes and purified by recentrifugation at the same density. The subnatant was collected through a hole pierced in the bottom of each tube. The density of this fraction was increased by the addition of solid sodium bromide to 1.063 g/ml followed by centrifugation for 48 hours at 37,000 rpm in a Ti 50 rotor. The LDL layer was then removed at the tops of the tubes by aspiration. The subnatant was again collected by piercing a hole in the bottom of the tubes. The density of these fractions was adjusted to 1.21 g/ml with solid sodium bromide and centrifuged for 48 hours at 37,000 rpm in a Ti 50 rotor. After removing the top layer containing the HDL, it was further purified by layering the HDL under a sodium bromide solution (d = 1.21 g/ml) and centrifugation as previously described. Chemical and electrophoretic characterization of each of the lipoprotein classes were carried out after dialysis against barbital buffer to remove the salt.

D. Phosphorus Determination

Lipid phosphorus was determined according to the method of Bartlett (92) with a modification as described by Parker et al. (93). The samples were placed in chromic acid-washed 10 ml conical centrifuge tubes, 0.25 ml of concentrated sulfuric acid was added, and placed in a sand bath heated to 250°C for 3 hours. The tubes were removed from the sand bath, allowed to cool and 3 drops of 30% hydrogen peroxide were added to each. The tubes were then heated for an additional 2 hours at 160°C. After cooling, 5 ml of water were added to the digestion mixture along with 0.6 ml of 2.5% ammonium molybdate. The contents were then mixed in a Vortex mixer and 0.24 ml of Fiske-Subba Row reagent was added and the tubes mixed again. The tubes were then placed in a boiling water bath, capped with a glass marble, and finally cooled to room temperature.

Colorimetric readings were taken in a Cary recording spectrophotometer (Model 11M, Applied Physics Company, Pasadena, Calif.) at 800 nm wavelength using quartz cells with a 1 cm light path.

The phosphorus content of the samples was obtained utilizing standard curves prepared from known amounts of potassium dihydrogen phosphate. Phospholipid concentrations were calculated by multiplying the phosphorus content by a factor of 25.4 which is based on an average molecular weight of 787 for egg yolk phosphatidylcholine (94).

E. Protein Determination

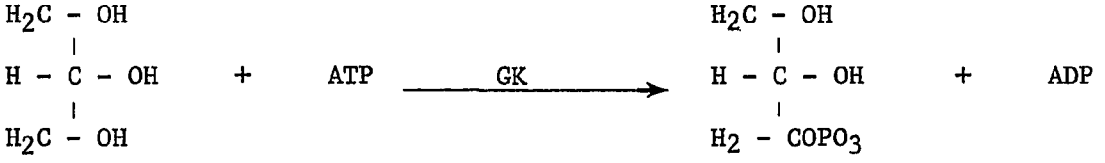
Protein was determined according to the method of Lowry (95). Protein samples were diluted to 0.25 ml with water and 0.25 ml of 2N NaOH was added. Five ml of a reagent consisting of 49 ml of 2% sodium carbonate, 1 ml of

1% sodium potassium tartarate, and 1 ml of 0.5% cupric sulfate were added and the contents mixed on a Vortex mixer. The optical density of the sample was measured at 750 nm using the Cary recording spectrophotometer 30 minutes after the addition of 0.5 ml of the Folin-Ciocalteau reagent previously diluted 1:1 with water. Standard curves were constructed for each assay using bovine serum albumin (Sigma Chemical Company, St. Louis, MO), dried over phosphorus pentoxide as the reference protein.

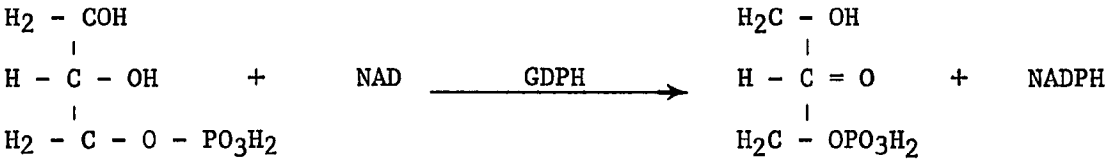
F. Triglyceride Determination

Triglycerides were determined according to the Hycel Triglyceride Test (Hycel Incorporated, Houston, Texas). Unlike classical methods of determining serum and lipoprotein triglycerides, this method utilizes a fungal lipase which specifically hydrolyzes triglycerides to glycerol and free fatty acids even in the presence of phospholipid. The glycerol is then measured through a series of enzyme reactions (96) coupled in a final step to the reduction to a tetrazolium salt forming a colored product easily measured colorimetrically. The principal reaction scheme is as follows:

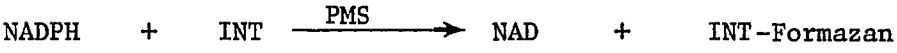
After the glycerol was formed via action of fungal lipase from triglyceride, the glycerol was phosphorylated by adenosive triphosphate (ATP) in the presence of glycerokinase (GK) to glycerol-1-phosphate (97):



The glycerol-1-phosphate was then dehydrogenated by nicotinamide adenine dinucleotide (NAD) to dihydroxyacetone phosphate. This reaction is catalyzed by glycerol phosphate dehydrogenase (GDPH) (98).



The NADH reduces iodonitritetrazolium violet (INT) to its colored formazan with phenozene methosulfate (PMS) as a catalyst.



The INT-Formazan end product was quantitated colorimetrically using the Cary recording spectrophotometer at a wavelength of 505 nm. A standard curve was constructed for each assay using normal, intermediate and elevated reference sera (Hycel Inc., Houston, Texas). When rabbit serum was measured, it was used as such. However, the accurate determination of lipoprotein triglyceride necessitated that the lipoprotein samples be first dialyzed against 0.005M Na veronal buffer, pH 7.4 in saline without EDTA since the presence of EDTA may inhibit enzyme activity. The actual procedure used is as follows: Samples of from 0.02 to 0.05 ml of serum or lipoproteins were pipetted into 1 x 8 cm test tubes and all volumes adjusted with distilled water to a total of 0.05 ml. At 20-second intervals, 0.5 ml of enzyme reagent was added. This reagent consisted of 1 part 2,900,000 U fungal lipase, 150,000 U glycerol phosphate dehydrogenase, and 1780 U glycerokinase to 50 parts 0.05% magnesium chloride, 8.9 mM adenosine triphosphate and 15.6 mM nicotinamide adenine dinucleotide. The tubes were mixed on a Vortex mixer and placed in a 37°C water bath. Exactly 10 minutes after the addition of the enzyme reagent 0.5 ml of 0.03% iodonitrotetrazolium violet and 0.005% phenazine methosulfate was added at 20 second intervals and the tubes were again placed in the 37°C bath for exactly 10 minutes. The tubes were then diluted with 2.0 ml of 0.1 N hydrochloric acid and cooled to room temperature.

The optical density of the samples was measured in the Cary recording spectrophotometer at 505 nm within 30 minutes after dilution. A standard curve was constructed for each determination using sera of known triglyceride composition as the reference lipid.

G. Cholesterol and Cholesteryl Ester Determination

Total serum and lipoprotein cholesterol were determined according to a method devised by Biodynamics/BMC (Boehringer-Mannheim, Indianapolis, Ind.). This procedure is based on the quantitative splitting of all cholesterol esters present in the sample into cholesterol and free fatty acids by the action of cholesterol esterase isolated according to Beauchamp et al. (99). In the presence of oxygen, free cholesterol is oxidized by cholesterol oxidase (isolated from Nocardia spp. according to Roeschlau et al. (100) to cholest-4-en-3-one):

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$

The hydrogen peroxide formed is used in the presence of catalase (from bovine liver - Boehringer-Mannheim, Indianapolis, Ind.) to transform methanol into formaldehyde:

$$H_2O_2 + CH_3OH \xrightarrow{\text{Catalase}} HCOH + 2H_2O$$

The formaldehyde reacts with ammonium ions and acetyl acetone in the Hantzsch (101) reaction to form 3,5-diacetyl-1,4-dihydrolutidine, which can be determined colorimetrically and is directly proportional to the cholesterol concentration.

The procedure for the total cholesterol determination was as follows: Serum or lipoprotein samples were pipetted into 1 x 10 cm test tubes, 5.0 ml of the cholesterol reagent mixture was added and the tubes mixed in a Vortex mixer. The cholesterol reagent mixture consisted of 10 parts 1.7 M methanol,

and 700 U/ml catalase (EC 1.11.1.6) in 0.6 M ammonium phosphate buffer, 0.5 parts 0.42 M acetylacetone and 2.1% w/v hydroxypolyethoxydodecase in 2.5 M methanol and 0.04 parts of 7 U/ml cholesterol esterase (EC 3.1.1.13). These tubes were designated as specimen blanks and a second set of specimen tubes was labeled. To this second set was added 0.02 ml of 4 U/ml cholesterol oxidase and 2.5 ml of the material from the specimen blank tubes. After thoroughly mixing all tubes, they were placed in a 37°C water bath for 1 hour. The tubes were then cooled and the optical density was determined using the Cary recording spectrophotometer at 410 nm. Cholesterol standards (Boehringer-Mannheim, Indianapolis, Ind.) were similarly treated and standard curves were constructed for each determination.

When free cholesterol was determined, the same procedure was carried out except for the omission of 0.04 parts of cholesterol esterase from the cholesterol reagent mixture. This technique prevented cholesteryl esters from being hydrolyzed thereby enabling only free cholesterol to be determined.

H. Dietary Regimes for the in Vivo Production of Unique VLDL

Twenty-four New Zealand white rabbits were bled via the left marginal ear vein and assayed for serum triglycerides, cholesterol and cholesteryl ester concentration. Twelve of these animals, whose serum lipids were in the middle range of values obtained, were maintained on a purina rabbit chow pelleted diet for 8-10 weeks and all experiments were performed. Next, a semisynthetic diet high in safflower oil (SAFF), was fed and the experiments repeated. After feeding the SAFF diet for 10 weeks, the chow diet was again fed for a period of two weeks and serum cholesterol determinations were made on each animal. These values were found to be within the normal

range as initially determined on the chow diet at this time. Lastly, a semisynthetic diet high in hydrogenated coconut oil (COCO) was fed. In this way, each animal served as its own control for the 8-10 week period. No exogenous dietary cholesterol was put in the last two diets. The composition of the individual diets is shown in Table 1.

Table 1. Composition of Experimental Diets

Constituent	% by Weight
Vitamin-free Casein	25.0%
Sucrose	40.0
Fat[1]	14.0
Salt Mix U.S.P. XIV	5.0
Cellulose (Solka-Floc)	15.0
Vitamin Mixture[2]	1.0

[1]The two experimental diets contained either 14% hydrogenated coconut oil, or 14% safflower oil.

[2]Amounts in gram/100 lbs. diet: Vitamin A (200,000 units/g), 4.5; Vitamin D (400,000 units/g), 0.25; α-tocopherol acetate, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-amino benzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0. In mg/100 lbs. diet: biotin, 20; folic acid, 90; Vitamin B_{12}, 1.35; D,L-methionine, 100.

The composition of the diets was taken from the catalog or feed tag of the commercial suppliers. The diets used were adequate in protein, carbohydrate, salt and vitamins (102). It should be noted that the chow diet contained a lower caloric density than the coconut or safflower oil diets but that the coconut and safflower oil diets were isocaloric with respect to one another. The fatty acid composition of the chow, COCO and SAFF diets is shown in Table 2.

The fatty acid composition of the Purina rabbit chow fat showed relatively high amounts of palmitic acid (13.2%), oleic acid (52.8%) and linoleic acid (22.3%).

The hydrogenated coconut oil semisynthetic diet fat contained lauric acid (47.3%), myristic acid (20.1%), palmitic acid (10.3%) and stearic acid (16.1%) as its major components with octadecenoic acid comprising 1.6% of the total fatty acid content.

The safflower oil diet fat contained palmitic acid (7.1%), stearic acid (2.7%), oleic acid (12.6%), and linoleic acid (77.0%). No attempts were made to characterize the location or isomeric form of the double bonds in the linoleic acid fraction.

I. Collection of Serum and Lipoprotein Lipase-Rich Post-Heparin Plasma

On the day of bleeding, each rabbit was weighed and placed in an animal restraining cage. A small portion of the animal's right ear was shaved with a scalpel blade over the area of a marginal ear vein. A nick approximately 1 cm in length was made longitudinally along the vein and the ear was placed inside a vacuum bleeding apparatus which was maintained at about 5-8 mm Hg below atmospheric pressure with a vacuum pump. Two ml of 0.25 M sodium citrate was used as an anticoagulant. In cases where serum

Table 2. Fatty Acid Composition of Dietary Lipids

Fatty Acid Chain Length	SAFF	COCO	Chow
8:0	-	0.6	-
10:0	-	4.0	-
12:0	-	47.3	1.4
14:0	-	20.1	1.9
16:0	7.7	10.2	13.2
16:1	-	-	2.9
18:0	2.7	16.0	3.4
18:1	12.6	1.6	52.8
18:2	77.0	0.2	22.3
18:3	-	-	2.1
Total Saturated Fatty Acids	10.4	98.2	19.9
P/S Ratio	7.4	.002	1.2

was collected, no anticoagulant was employed and the blood merely allowed to clot. Blood samples (40 ml) collected in this fashion were immediately placed on crushed ice and centrifuged at 1200 g for 40 minutes in order to obtain either serum or plasma.

When LPL-rich post-heparin plasma (PHP LPL) was to be collected, 100 units sodium heparin/kg body weight were injected intravenously into a marginal ear vein of the left ear and blood was collected from a marginal ear vein of the right ear after 5 minutes.

J. Collection of Extra-Hepatic Post-Heparin Plasma

Twenty-four hours later the same rabbit as above was used as a source of LPL-rich plasma of extra-hepatic origin. This plasma was collected and prepared essentially by the method of Bezman-Tarcher and Robinson (103). Adult New Zealand white rabbits weighing approximately 3.5 kg were preanesthetized with 0.02 mg/lb. atropine sulfate (Pitman Moore, Washington Crossing, N.J.), given subcutaneously. After 10 minutes, 0.1 mg/lb. acepromazine maleate (Ayerst Labs, New York, N.Y.) and 10 mg/lb. ketamine hydrochloride (Bristol Labs, Syracuse, N.Y.), were given intramuscularly to induce anesthesia. A surgical plane of anesthesia and analgesia was obtained after 5-8 minutes and a ventral midline incision was made in order to dissect the abdominal aorta just proximal to the celiac artery and to place one loose ligature of 1/8" umbilical tape around it. Next the post vena cava was dissected distil to the diaphragm and proximal to the liver and another loose ligature placed around it. Then a 2-3" section of the inferior vena cava, at the level of the left renal vein, was dissected and, using umbilical tape and a short length of polyethylene tubing, both the distil and proximal parts of the post cava were temporarily ligated, stopping venous flow in

the area. A polyethylene cannula was then introduced into this part of the inferior vena cava and it was tied in place at the level of the right atrium. Heparin solution (100 U/kg body weight) was introduced through this cannula and the rabbit was exsanguinated five minutes later through the abdominal aorta. Serum collected in this fashion was used for the partial purification of extra-hepatic lipoprotein lipase.

K. Preparation of Partially Purified LPL (Triacylglycerol Hydrolase)

The densities of post-heparin plasma, extra-hepatic post-heparin plasma, and normal plasma were adjusted to 1.063 and centrifuged at 40,000 rpm for 36-48 hours in a Beckman Ti-50 rotor in a Beckman L3-50 preparative ultracentrifuge (Beckman, Inc., Palo Alto, Calif.). This was done to disrupt the VLDL-LPL complexes and to remove all lipoproteins of density less than 1.063 g/ml. The top layer which contained these lipoproteins was removed and discarded. The subnatant was assayed for LPL activity.

L. Assay of Triacylglycerol Hydrolase (Lipoprotein Lipase)

1. Preparation of Triglyceride Emulsions and Assay

Artificial substrate emulsions for the assay and characterization of rabbit triacylglycerol lipases were prepared essentially according to the method of Nilsson-Ehle et al. (104). Radio-labeled H^3-triolein and unlabeled triolein were mixed to give 100 mg of triolein with about 1.36×10^6 cpm and 6.0 mg of egg phosphatidyl choline was added. After evaporating the solvent under a stream of nitrogen gas, the dried lipids were emulsified in 1.0 ml (1.25 g) of glycerol by homogenization for 5 minutes using a Branson Model J-18A Sonicator equipped with a micro-probe (Branson Sonic Power Co., Danbury, Conn.).

The homogenization was carried out in a glass screw-capped vial, 1 x 6 cm, immersed in an ice bath. The emulsion is optically clear and could be stored at 20°C with no auto-hydrolysis of triglyceride for up to about 6 weeks (as determined by thin-layer chromatography).

Substrate solutions for assay were prepared fresh as needed by combining 0.2 ml of the concentrated substrate emulsion described above with 0.8 ml 0.005 M sodium barbital buffer, pH 7.4 containing 3% bovine serum albumin and 0.2 ml of normal rabbit serum. This mixture was vigorously shaken on a vortex mixer for 30 seconds and yielded an opaque emulsion ready for use. Incubations were carried out in 10 ml screw-capped conical centrifuge tubes at 37°C in a total volume of 0.2 ml with 0.09 ml of substrate, 0.01-0.06 ml of enzyme preparation, and 0.05-.10 ml 5 mM sodium barbital buffer in saline, pH 7.4. Other conditions are as indicated in Results. The final concentration in the assay tubes were triolein, 6.1 μmoles/ml; lecithin, .51 μmole/ml; albumin, 1% (w/v); and serum, 9% (v/v); in 5 mM sodium barbital buffer, pH 7.4 in saline. Assay time was typically 1 hour.

The fatty acid produced during the incubation period was isolated via a modification of the liquid-liquid partition system described by Belfrage and Vaughan (105). The reaction was stopped by adding 3.3 ml of methanol: chloroform:heptane (1.41:1.25:1) v/v/v, followed by 1.0 ml of 0.1 potassium carbonate-borate buffer, pH 10.5. The extraction and all other subsequent procedures were performed at room temperature. After thoroughly mixing each tube in a vortex mixer for 15 seconds, the tubes were centrifuged for 25 minutes at 1100 x g. A 1.0 ml aliquot of the methanol-water upper phase (total volume of upper phase 2.4 ml) was counted in a Packard Tri-Carb

Liquid Scintillation Spectrometer using 10 ml of Aquasol (New England Nuclear, Boston, Mass.). This upper phase contained about 76% of the H^3-oleic acid released. An aliquot (10 µl) of the assay substrate was assayed for radioactivity after the addition of 1 ml of upper phase from control incubations without enzyme.

2. Acetone Injection Method for Radio-Labeling of VLDL-Triglyceride

The desired amount of H^3-9,10 triolein was dissolved in 10-15 µl of acetone. The labeled triolein was then slowly injected into 1.0 ml of rapidly stirring VLDL in 5 mM sodium barbital buffer, pH 7.4, under a steady stream of nitrogen gas which helped remove the acetone. Next 50 mg of bovine serum albumin per ml of VLDL was added, the mixture was equilibrated for 12 hours at 4°C and then centrifuged for 30 minutes at 37,000 rpm in a 40.3 rotor to remove non-incorporated aggregates.

3. Assay with Natural Substrate

One microliter of the enzyme preparation to be measured was added to 80 µl of radio-labeled VLDL (3.00 mg triglyceride/ml) in 5 mM sodium barbital buffer, pH 7.4, in saline to a final assay volume of 0.2 ml (final assay concentrations were 1.2 mg triglyceride/ml, 5 mM sodium barbital in 0.9% NaCl, pH 7.4, and approximately 50-70 mUnits/ml lipase activity as determined under artificial substrate assay conditions). The assay was performed at 37°C for 30 minutes as the fatty acids released were extracted and counted in a Packard Tri-Carb Scintillation Spectrometer as described above for the artificial substrate assay method.

M. The Selective Measurement of Extra-Hepatic Triacylglycerol Hydrolase Activities

After the intravenous injection of sodium heparin, it is possible to measure, in plasma, the activities of 2 different triacylglycerol hydrolases: lipoprotein lipase released from peripheral tissues and hepatic lipase. These activities were measured according to the method of Krauss et al. (53) which takes advantage of the selective inhibition of the peripheral enzyme activity in the presence of protamine sulfate.

Typically, 10 µl aliquots of PHP LPL were preincubated with from 0.2 to 7.2 mg protamine sulfate/ml final assay mixture for 10 minutes at 27°C. Next, 60 µl of the radio-labeled artificial substrate emulsion was added in a final concentration of 3 mg triolein/ml assay mixture and the tubes were incubated at 37°C for 1 hour. The free fatty acids released were then extracted (see Assay of LPL in Materials and Methods section) and counted in the Packard Tri-Carb Scintillation Spectrometer. Results of this experiment provided a measure of the protamine resistant triacylglycerol hydrolase activity in the PHP LPL preparation.

Similarly, aliquots of PHP LPL were pre-incubated in 5 mM barbital buffer for 10 minutes at 27°C, then assayed for lipase activity in order to measure the total triacylglycerol hydrolase activity. Protamine sensitive lipase activity was then measured by subtraction of the two values obtained.

N. Agarose Electrophoresis of Lipoproteins

Agarose gel electrophoresis of lipoproteins or sera was carried out utilizing the ACI Cassette Electrophoresis Cell and power supply having a constant output of 90V (Corning Medical, Palo Alto, Calif.). Agarose

Universal electrophoresis film (Analytical Chemists, Inc., Palo Alto, Calif.) was used, which are pre-cast gels consisting of 1.0% agarose, 5% sucrose and 0.035% disodium EDTA in 0.065 M barbital buffer, pH 8.6. Lipoprotein or serum samples of 1.0 to 3.0 µl were applied to the sample wells in the gel and allowed to evaporate at room temperature. Next the agarose film was placed into the cassette cover and placed on the cell which was filled with 0.05 M barbital buffer, pH 8.6, containing 0.035% EDTA. The samples were allowed to migrate for 30 minutes.

After electrophoresis, the agarose film was heat-dried in a 75°C oven to fix the lipoprotein. The lipoproteins were then stained with 0.02% fat Red 7B, clearing and rinsing the film sequentially in MeOH:H_2O (1:1 v/v) and 2% glycerol or in 10% acetic acid. The gels were then quantitated densitrometrically on a Gelman ACD 15 Model 39430 Scanning Densitometer (Gelman Instrument Co., Ann Arbor, Mich.) at 575 nm.

O. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out using a Bio-Rad Model 220-vertical slab gel electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.). Separating gels, 14% in acrylamide, were cast between 14 x 17 cm glass plates containing 0.1% sodium dodecyl sulfate in 1.5 M tris HCl buffer, pH 8.4, using 0.05% ammonium persulfate as a polymerizing agent. A stacking gel consisting of 5% acylamide was next cast using the plastic comb provided by Bio-Rad for the purpose of making sample wells at the top of the vertical slab. Samples were prepared by mixing equal volume amounts of from 10-60 µg lipoprotein with a solution containing 20 ml 10% SDS, 5 ml mercaptoethanol, 10 mg glycerol and 12.5 ml of 0.5 M

tris HCl buffer pH 6.8 to which 0.01% Bromphenol blue was added. This mixture was then heated for 10 minutes at 100°C in order to solubilize the apoproteins of the specific lipoprotein used.

The apoproteins were electrophoretically separated at a constant current of 9 mA for 3-4 hours or until the Bromphenol blue tracking dye was 1.0 cm from the bottom of the slab.

Gels were removed from between the glass plates and stained overnight in 0.025% Coomassie blue in isopropanol:H_2O:acetic acid (25:65:10) and destained for 12 hours in 10% acetic acid. The gels were then sealed in 10% acetic acid in optically clear plastic film using a Dazey Seal-a-Meal Model SAM 1 (Dazey Products Co., Industrial Airport, Kans.), and stored until densitometric tracings could be accomplished. Scans were conducted with the Gelman Densitometer at 675 nm.

P. Preparation of VLDL Remnant Lipoproteins

After enzyme preparations, NPLAS, PHP LPL, and EHP LPL were isolated ($d > 1.063$ g/ml), VLDL which contained 0.9 mg triglyceride/ml VLDL was incubated in rotor Ti 50 cellulose nitrate tubes with these enzyme preparations containing lipase activity of approximately 17.5 µmoles free fatty acid released/hour for 4 hours, in 0.005 M sodium barbital buffer, pH 7.4 in saline. The incubation was stopped by adding enough NaCl of density 1.1923 g/ml to adjust the media to a density of 1.019 g/ml. The tubes were centrifuged at 48,000 rpm in a Ti 50 rotor for 24 hours. The supernatant resulting from this centrifugation was designated VLDL remnants and was further purified by recentrifugation in the Ti 50 rotor at 48,000 rpm for 48 hours in a Beckman L3-50 or L2-65B preparative ultracentrifuge.

Q. Total Lipid Extraction of Serum Lipoproteins

To tubes containing 0.1 ml of lipoprotein (ca 3-5 mg/ml), 2.0 ml of $CHCl_3$:MeOH 2:1 with 0.2 µl acetic acid per 100 ml were added. The tubes were shaken on a mechanical shaker for 15 minutes, heated in a 60°C water bath for 15 minutes and again shaken for 15 minutes. After centrifugation at 2500 rpm in a clinical centrifuge for 5 minutes, 0.5 ml H_2O was added and the tubes were again shaken and centrifuged for 15 minutes each and the aqueous phase was removed and discarded. The organic phase containing the lipid was dried under a steady stream of nitrogen gas and 10 µl of $CHCl_3$:MeOH (2:1, v/v) was added to each tube.

R. Thin-Layer Chromatography of Lipoprotein Lipids

Major lipid classes were fractionated via thin-layer chromatography using silica gel G coated glass plates, 20 x 20 cm, which had been activated for 1 hour at 110°C. After applying the lipid samples and appropriate standard lipids under a stream of nitrogen gas to the plates, they were developed in hexane:ether:acetic acid 90:10:1. The solvent was allowed to dissipate in a specially constructed plexiglass box which allowed a constant flow of nitrogen gas across the plate in order to protect the lipid from oxidation. Next the fractionated lipids were briefly visualized under iodine vapor, the spots outlined using a clean dissecting needle, and the plate returned to the nitrogen gas flow box to get rid of any residual iodine. The spots were then scraped off the plates and placed in 1.5 x 13 cm screw-capped test tubes fitted with teflon liners.

S. Preparation of Fatty Acid Methyl Esters of Lipid Classes

To the tubes from the above procedure (containing fractionated lipid classes and silica gel) 2 ml of 4% sulfuric acid in methanol were added. The tubes were then heated for 60 minutes on a steam cone. After cooling, 2 ml of distilled water were added, and the methyl esters of the fatty acids were extracted by three separate additions of 2 ml hexane, the contents mixed on a vortex mixer and the upper layer was pooled. Residual water was removed from the hexane phase by addition of solid anhydrous sodium sulfite. Controls of reagents and silica gel from developed plates were routinely run simultaneously with lipid samples.

T. Analysis of Fatty Acyl Composition of Lipid Classes by Gas-Liquid Chromatography

The methyl esters in hexane were concentrated under a stream of nitrogen gas for analysis by gas-liquid chromatography. Fatty acid compositional analysis was performed on a 6' x 1/8" stainless steel column, packed with 10% SP2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Penn.). Fatty acids were identified by GLC analysis of commercially available methyl esters of fatty acid standards by equating retention times.

U. Gas-Liquid Chromatography Operation Conditions

The analysis of methyl esters of fatty acids from phospholipid, triglycerides and cholesteryl esters was performed on a Hewlett Packard Research Gas Chromatography Model 5750 equipped with a dual flame ionization detector. The injection port heater and the flame ionization detector heater were maintained at 250°C and 290°C respectively. The operating conditions consisted of 30 ml/minute helium carrier gas flow rate measured

at the column outlet, with 30 ml/minute hydrogen gas flow rate, and 250 ml/minute air flow rate. Column oven temperature was maintained at 190°C and injections were performed directly into the column. The chromatograph electrometer was interfaced with a Hewlett Packard Digital Integrator Model 3380A which produces a chromatogram and determines the retention times of peaks, peak area and percentage of each peak area of the total. The integrator was started simultaneously with sample injection. Throughout the analysis, the chromatograph electrometer was operated at range 100 and attenuation 2.

V. Source of Chemicals

Triolein (>99.5% pure) and methyl ester standards for gas-liquid chromatography were purchased from NuChek Prep, Inc., Elysian, Minnesota. Glycerol tri (9,10(n)-H^3) oleate, (H^3-triolein), 50 mCi/mmole was supplied by Amersham Corporation, Arlington Heights, Illinois. Pre-cast 1% agarose slab gels were purchased from ACI/Corning, Palo Alto, California, and all polyacrylamide gel reagents were obtained from Bio-Rad Laboratories, Richmond, California. Safflower oil for the diets was purchased from a local supplier, manufactured by Hain Pure Food Company, Los Angeles, California, while the hydrogenated coconut oil was obtained from Drew and Company, New Jersey. Casein was purchased from Erie Casein Company, Erie, Illinois, and Salt Mixture XIV and the Vitamin Diet Fortification Mixture were obtained from the Nutritional Biochemicals Division of ICN Life Science Group, Cleveland, Ohio. Additionally, after the diets were mixed, they were pelleted by the ICN Life Science Group, Cleveland, Ohio. Protamine sulfate (from salmon, grade 1) and bovine serum albumin, essentially fatty acid free were purchased from Sigma Chemical Company, St. Louis,

Missouri, while sodium heparin (100 units/mg) was obtained from Nutritional Biochemicals, Cleveland, Ohio. The triglyceride test set was supplied (Hycel #961) by Fischer Scientific Company, Itasca, Illinois. The cholesterol reagent test set was obtained from Bio-dynamics Division of Boehringer-Mannheim, Indianapolis, Indiana. All other chemicals and reagents were supplied by Fischer Scientific Company, Fairtown, New Jersey, or Mallinckrodt Chemical Works, St. Louis, Missouri.

III. RESULTS

Section I

A. Adequacy of the Dietary Fatty Acids

To assure that the hypercholesterolemia and altered VLDL produced by the COCO diet was not secondary to essential fatty acid deficiency, the fatty acid composition of the lipoprotein phospholipids were analyzed. Holman has found that a triene:tetraene (20:3ω9:20:4ω6) ratio greater than 0.4 is seen in EFA-deficient rats (106). Likewise a ratio of oleic acid to linoleic acid greater than 1.5 is indicative of EFA deficiency in the phospholipid fraction of guinea pig erythrocytes (107). None of the VLDL phospholipids of rabbits analyzed had a triene:tetraene ratio greater than 0.28 or an oleic acid:linoleic acid greater than 1.13. There was some weight loss in a few of the animals during the first ten days on both the COCO and SAFF diets but the weight was regained by the end of the third week. By these criteria, then, the stores of EFA in these adult animals was adequate to protect them from any deficiency over the course of the feeding study employed.

B. Serum Lipid and Lipoprotein Profiles: Variation with Diet

1. Serum Lipid Concentration

The effect of the diets on serum triglyceride, total cholesterol, and free and esterified cholesterol levels is seen in Table 3. The cholesterol free, high fat semisynthetic diets produce a marked cholesterolemia in the rabbits fed these diets for 9 weeks. The SAFF diet-fed animals' serum cholesterol values were also elevated when compared to the chow-fed case, but the differences here were not as statistically significant as when the COCO diet was fed. Serum triglyceride levels in the animals were relatively unchanged except in the SAFF case where a slight decrease in the triglyceride concentration was observed. Additionally, the serum of the rabbits after feeding the COCO diet was noticeably lipemic.

2. Plasma Lipoprotein Concentration

Rabbits fed both experimental diets showed elevated concentrations of VLDL (Table 4), especially in the case of the COCO diet. With respect to the LDL concentration ($LDL_1 + LDL_2$), the COCO diet yielded an increased amount of this fraction while the SAFF diet resulted in a somewhat lower concentration as compared to the chow-fed case. HDL values, while not too significantly different among the three dietary treatments, showed a decreasing trend in the experimental groups, with the COCO-fed rabbit HDL having the lowest HDL concentration. The most significant finding is that when the experimental diets are fed, an increase in concentration of serum lipoproteins with $d < 1.063$ g/ml occurs. In the COCO diet-fed case, more than 93% of the serum lipoproteins exist in this $d < 1.063$ g/ml fraction. Serum lipoproteins from SAFF- and chow-fed animals are more similar to

Table 3. Serum Triglyceride (TG), Total Cholesterol (TC), Free Cholesterol (FC), and Cholesteryl Ester (CE) of Rabbits after 9 Weeks on Different Diets (mg/dl±S.E.M.)

Diet	TG	FC	CE*	TC
SAFF (n=10)	67±7[a]	50±8[a]	218±23[a]	178±22[a]
COCO (n=10)	113±20	135±17[b,d]	537±55[b,e]	451±42[c,e]
Chow (n=9)	104±10	30±2	151±11	119±8

[a]Significantly different from chow @ $p < .05$ level

[b]Significantly different from chow @ $p < .01$ level

[c]Significantly different from chow @ $p < .001$ level

[d]Significantly different from SAFF @ $p < .05$ level

[e]Significantly different from SAFF @ $p < .01$ level

*Cholesteryl ester deterimined as free cholesterol, but expressed as cholesteryl oleate

Table 4. Concentration of Serum Lipoproteins in Response to Different Diets (mg/dl)

Diet	VLDL n=10	LDL_1 n=5	LDL_2 n=5	HDL n=5	LDL/HDL	Total
SAFF	40±9[a]	12±1[c]	39±3	24±2	2.1	115
COCO	144±5[b,d]	4±0.7	120±5[d]	20±2[e]	6.2	288
Chow	15±1	n/d	85±2	27±2	3.1	127

[a]Significantly different from chow @ $p < .01$ level

[b]Significantly different from chow @ $p < .001$ level

[c]Significantly different from COCO @ $p < .01$ level

[d]Significantly different from SAFF @ $p < .001$ level

[e]Significantly different from chow @ $p < .05$ level

n/d - Not determined

one another in terms of distribution of density fractions. The LDL to HDL ratio, which has been used as a measure of atherogenicity, is also included in Table 4 and in the case of the COCO-fed rabbits is appreciably elevated when compared to the two other dietary treatments.

3. Summary of Lipoprotein Alterations

Five major changes are consistently observed in the serum lipoproteins after cholesterol feeding (see Literature Review). Table 5 summarizes the results of feeding the two semisynthetic diets on these five parameters and compares them with the cholesterol-induced hypercholesterolemic case. In addition to the hypercholesterolemia seen in rabbits fed the COCO diet, only four of these five major changes occurred. The presence of B-VLDL as well as increases in IDL and LDL and a decrease in the typical HDL were observed which are similar to the result reported in cholesterol-induced hyperlipoproteinemia (60). No detectible amount of HDL_c was found to be present, however.

In the SAFF-fed case, on the other hand, while an increase in serum cholesterol and IDL and a decrease in typical HDL were observed, no B-VLDL or HDL_c were noted, and LDL decreased somewhat.

C. Chemical Composition of Serum Lipoproteins: Variation with Diet

1. VLDL

Figure 1 shows the chemical composition of the rabbit serum VLDL from each dietary group. In the experimental diets, the rabbit VLDL was changed from triglyceride-rich to cholesteryl ester enriched particles. The triglyceride-rich VLDL (59.7%) from chow-fed rabbits averaged 11.9% protein, 18.8% phospholipid, 3.1% free cholesterol and 8.2% cholesteryl ester. The

Table 5. The Effect of High Saturated or Unsaturated Fat Diets and High Cholesterol Supplementation on the Serum Lipoprotein Profile: Comparison of Dietary Treatments

Lipoprotein	Dietary Treatment		
	Cholesterol Induced[1]	COCO Diet	SAFF Diet
B-VLDL	Yes	Yes	No
IDL	↑	↑	↑
LDL	↑	↑	↓
HDL_c	Yes	No	No
HDL_2	↑	↓	↓

B-VLDL refers to that lipoprotein with d < 1.006 g/ml and with β-mobility on agarose or paper electrophoresis

HDL_c refers to that lipoprotein which has characteristics in common with both LDL and the typical HDL

HDL_2 refers to that lipoprotein regarded as typical HDL

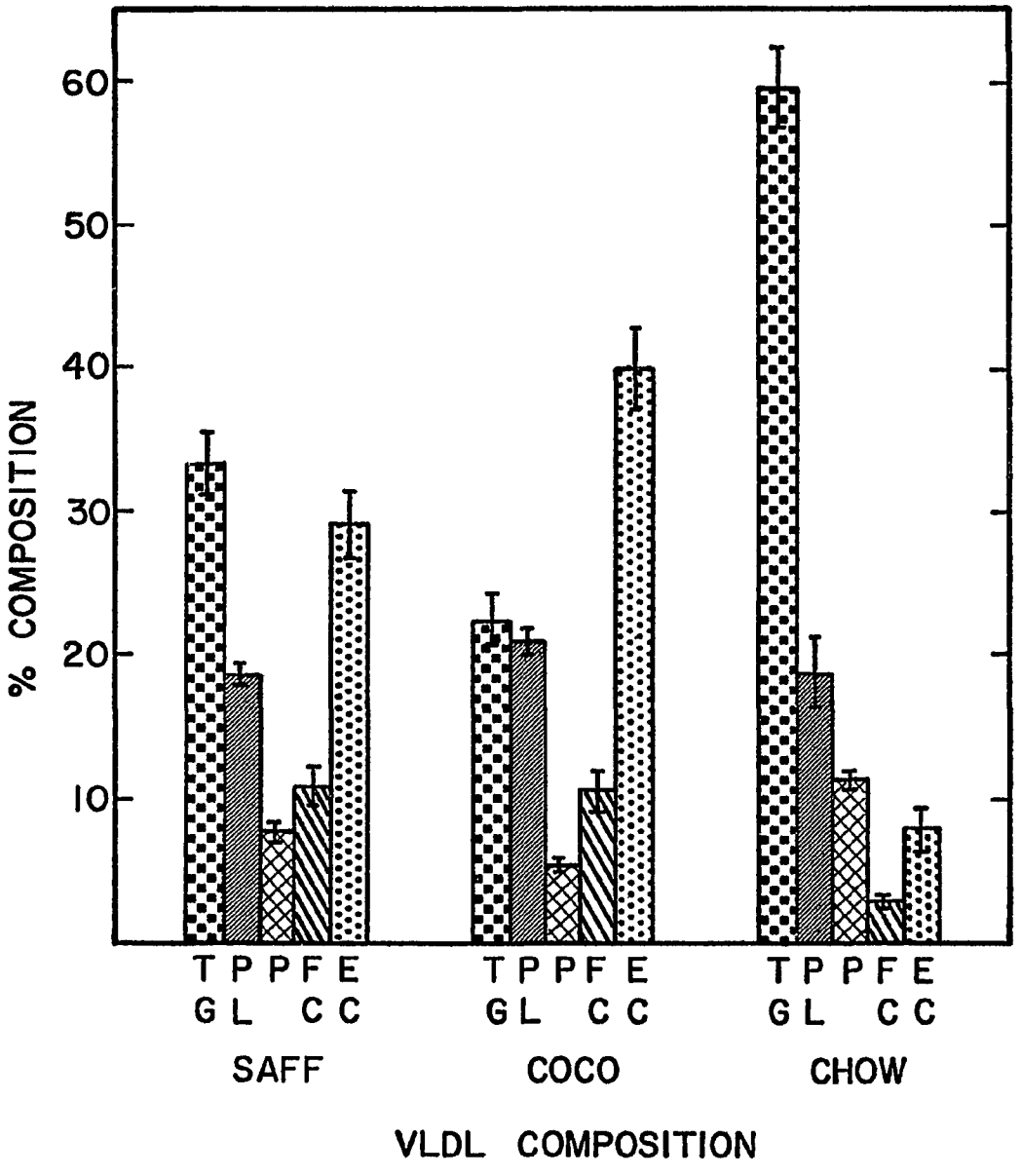
[1]From Disturbances in Lipid and Lipoprotein Metabolism, 1978, eds. J.M. Dietschy, A.M. Gotto, Jr., J.A. Ontoko, Baltimore: Waverly, p. 188.

↑ refers to increased serum concentration

↓ refers to decreased serum concentration

yes/no refers to presence or absence of this lipoprotein

Figure 1. Relative chemical composition of rabbit serum VLDL from each dietary group. The abbreviations used are as follows: SAFF, semisynthetic rabbit diet containing 14% safflower oil; COCO, semisynthetic rabbit diet containing 14% hydrogenated coconut oil; chow, normal chow diet; TG, triglyceride; PL, phospholipid; P, protein; FC, free cholesterol; EC, esterified cholesterol. Standard errors are indicated for n = 10.

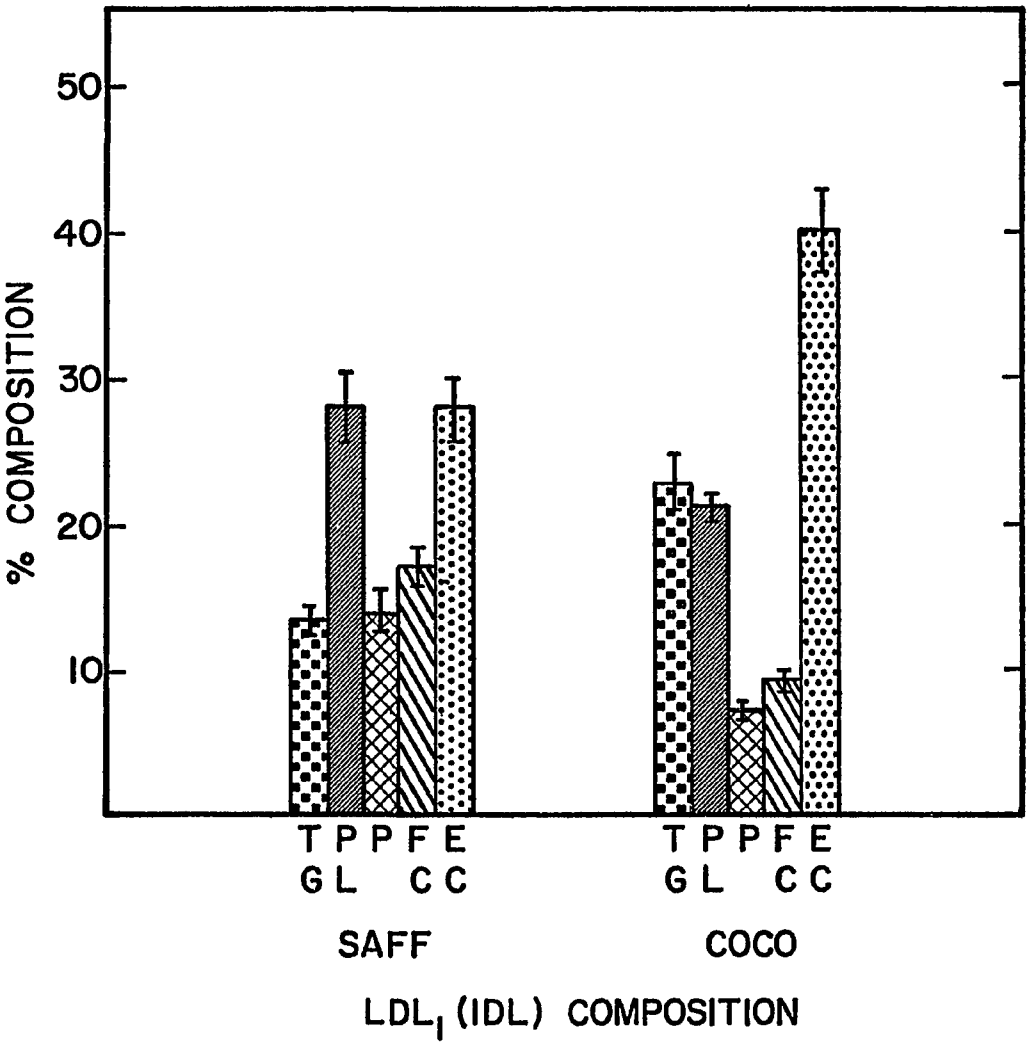

60
50
40
30
20
10
% COMPOSITION
T P P F E
G L C C
T P P F E
G L C C
T P P F E
G L C C
SAFF
COCO
CHOW
VLDL COMPOSITION

cholesteryl ester enriched VLDL from COCO-fed animals contained only 22.5% triglyceride, 5.4% protein, 21.2% phospholipid, 10.8% cholesterol and 40.1% cholesteryl ester. The chemical composition of VLDL from animals fed the SAFF diet were in between the two other groups in terms of triglyceride, protein and cholesterol content with 33.3% triglyceride occurring along with 7.7% protein, 18.8% phospholipid, 11.0% free cholesterol and 29.2% esterified cholesterol. Phospholipid values did not differ markedly between the three groups, yet the protein to lipid ratios of chow, COCO, and SAFF diet-fed rabbits were 0.10, 0.06 and 0.08 respectively. In addition, the surface to core ratio (P + PL + C/TG + CE) were .50, .60, and .60 for chow, COCO, and SAFF diet-fed animals respectively.

2. LDL_1 (IDL)

Figure 2 shows the composition of the rabbit serum LDL_1 (IDL) found after the feeding of the two experimental diets used. In the case of the COCO-fed animals, more triglyceride and cholesteryl esters are carried in this intermediate density fraction ($1.006 < d < 1.019$ g/ml). SAFF diet-fed rabbit IDL contained 13.4% triglyceride, and 27.8% cholesteryl ester with 13.9% protein, 27.8% phospholipid, and 17.1% free cholesterol. In the animals fed the COCO diet, it was found that the IDL fraction was made up of 22.6% triglyceride and 39.8% cholesteryl ester with surface constituents in the following proportions: 7.3% protein, 21.2% phospholipid and 9.1% free cholesterol. The sum of the core components (i.e., triglyceride and cholesteryl esters) from the COCO diet-fed rabbit IDL indicates that this dietary regimen yielded an intermediate density particle which is perhaps larger than its SAFF diet counterpart. That is, the contribution of core constituents in the case of IDL from the COCO diet-fed rabbit is

Figure 2. Relative chemical composition of rabbit serum IDL (LDL_1) from each dietary group. The abbreviations used are as described in Figure 1. Standard errors are indicated for n = 5.


50
40
30
20
10
% COMPOSITION
T G
P L
P C
F C
E C
T G
P L
P C
F C
E C
SAFF
COCO
LDL$_1$ (IDL) COMPOSITION

much greater than in the SAFF diet-fed case. Surface to core ratios also are consistent with this idea with the COCO and SAFF IDLs having values of 0.6 and 1.4 respectively.

3. LDL_2

The chemical composition of rabbit serum LDL_2 ($1.019 < d < 1.063$ g/ml) from the animals fed the various diets is indicated in Figure 3. The values obtained were as follows: SAFF diet LDL_2: triglyceride, 9.6%, cholesteryl ester, 17.7%, with 18.9% protein, 32.1% phospholipid, and 21.6% free cholesterol; COCO diet LDL_2: triglyceride, 9.2%, cholesteryl ester, 7.2%, with 25.6% protein, 44.1% phospholipid and 14.0% free cholesterol; chow diet LDL_2: triglyceride, 12.7%, cholesteryl ester, 24.6% with 33.5% protein, 23.5% phospholipid and 6.3% free cholesterol. Figure 3 also shows that there is a large significant increase in the relative amount of phospholipid and free cholesterol in the case of the experimental diet serum LDL_2 as compared to the chow-fed LDL_2 case. Surface to core ratios for LDL_2 from each of the three dietary treatments are 2.7, 5.1, and 1.7 for the SAFF, COCO, and chow-fed animals' LDL_2 respectively, indicating a quite small particle in the COCO case as compared to the LDL_2 for the other two dietary treatments.

4. HDL

The composition of HDL fractions ($1.063 < d < 1.21$ g/ml) of animals fed the various diets are found in Figure 4. Chow-fed rabbits contained 7.2% triglyceride, 3.1% cholesteryl ester along with 47.2% protein, 30.9% phospholipid and 3.1% free cholesterol in the HDL. In the SAFF diet-fed animal, triglyceride comprised 2.1%, cholesteryl ester, 26.5%, protein,

Figure 3. Relative chemical composition of rabbit serum LDL (LDL_2) from each dietary group. The abbreviations used are as described in Figure 1. Standard errors are indicated for n = 5.

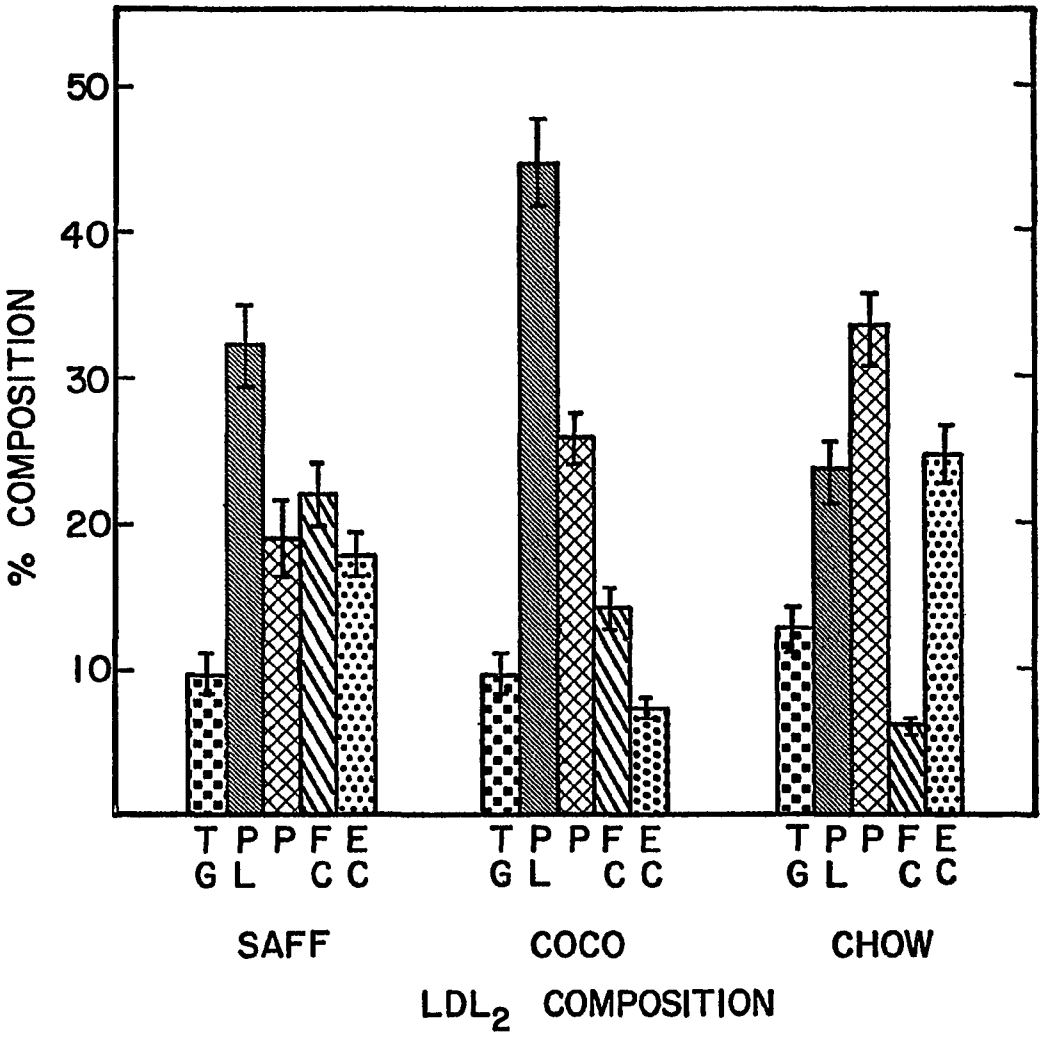
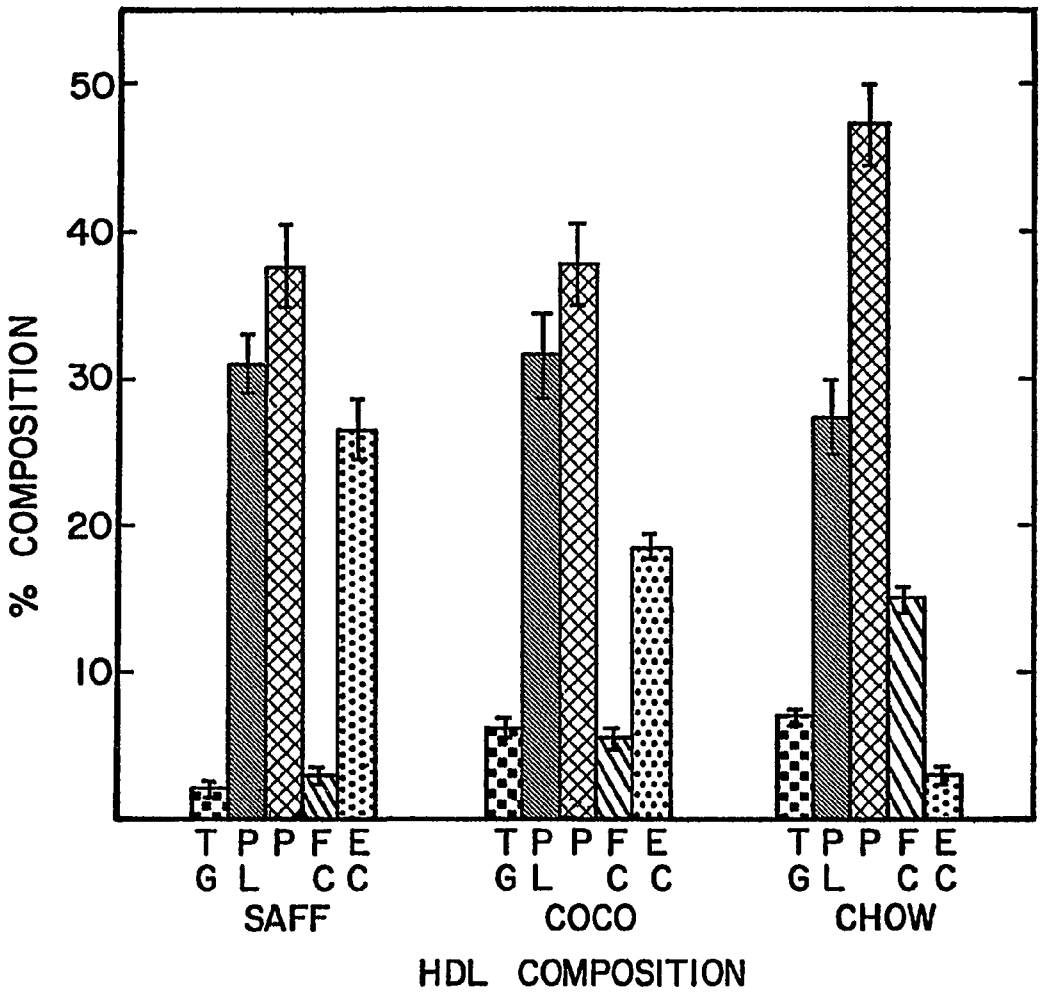

50
40
30
20
10
% COMPOSITION
TG
PL
PC
FC
EC
SAFF
COCO
CHOW
LDL2 COMPOSITION

Figure 4. Relative chemical composition of rabbit serum HDL from each dietary group. The abbreviations used are as described in Figure 1. Standard errors are indicated for n = 5.


50
40
30
20
10
% COMPOSITION
TG
PL
PP
FC
EC
SAFF
COCO
CHOW
HDL COMPOSITION

37.5%, phospholipid, 30.9% and free cholesterol, 3.1%. The COCO-fed case yielded HDL of the following composition: triglyceride, 6.3%, cholesteryl ester 18.5% with 37.8% protein, 31.7% phospholipid, and 5.6% free cholesterol. This fraction appears to be preferentially enriched in cholesteryl esters at the expense of a decrease in free cholesterol, triglyceride and protein. Surface to core ratios for this fraction are 2.5 for the SAFF-HDL, 3.0 for the COCO-HDL, and 8.7 for the chow-HDL, indicating that the HDL from experimental diets are much larger particles when compared to the chow case. This finding is consistent with an HDL particle relatively enriched in its core cholesteryl ester component and is in agreement with the concept that HDL may be attempting to protect against cellular cholesterol accumulation.

D. Electron Microscopy of VLDL

Electron microscopy of the isolated VLDL was performed in order to check the heterogeneity and mean particle size of the VLDL preparations. Figure 5 shows the distribution of VLDL sizes typically found. The chow-fed VLDL had a mean diameter of 365 Å. This finding is consistent with previously reported VLDL sizes (108) and was used to assess the presence of VLDL in the $d < 1.006$ g/ml fraction isolated by ultracentrifugation.

E. Agarose Gel Electrophoresis of Serum Lipoproteins: Variation with Diet

The results of typical densitometric scans of serum after agarose electrophoresis from rabbits fed the various diets, stained with fat red 7B, are seen in Figure 6. Serum from rabbits fed chow or SAFF diets yielded the normal pattern of beta, pre-beta, and alpha lipoproteins. However, when the COCO diet was fed, electrophoresis of the serum lipoproteins showed the

Figure 5. Distribution of chow-fed rabbit VLDL particle diameters in angstrom units.

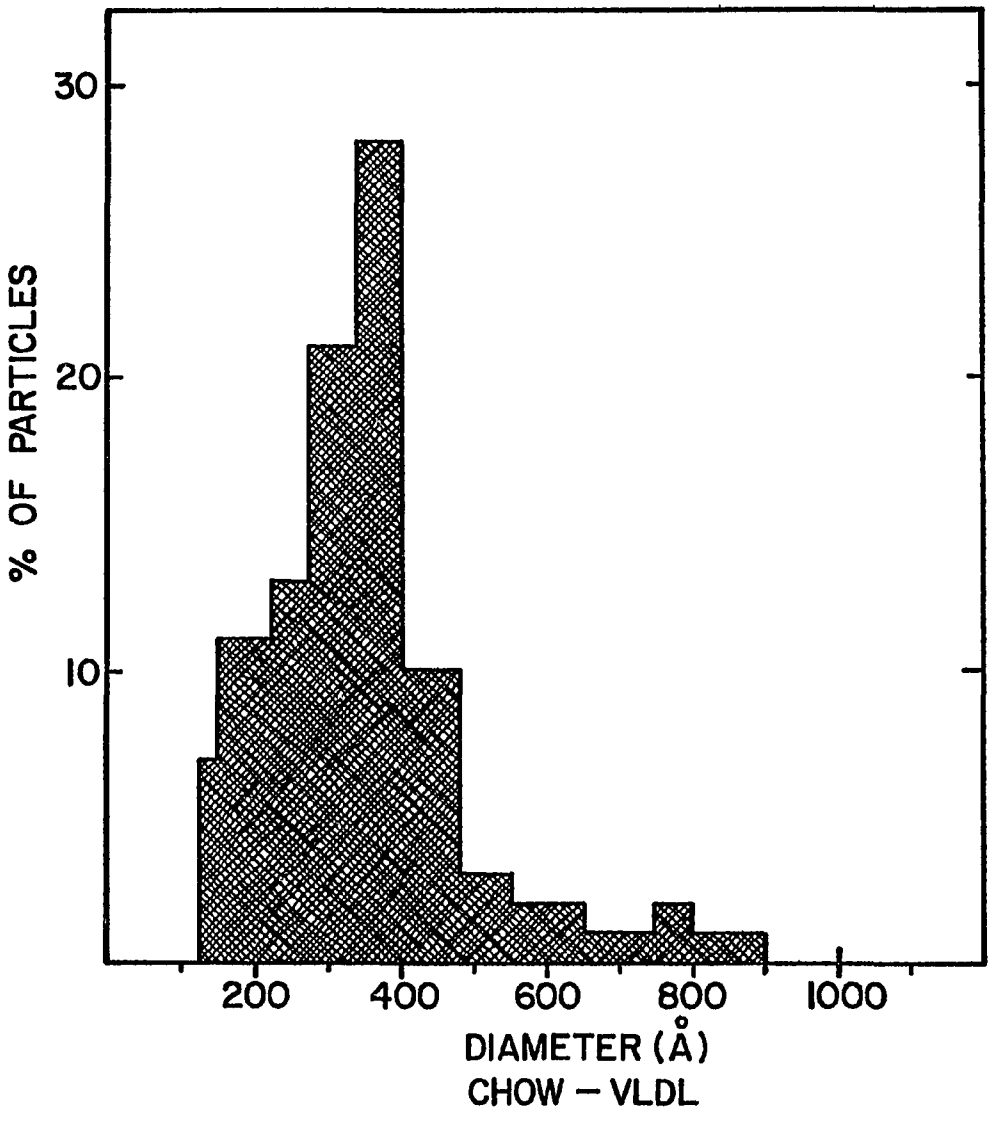
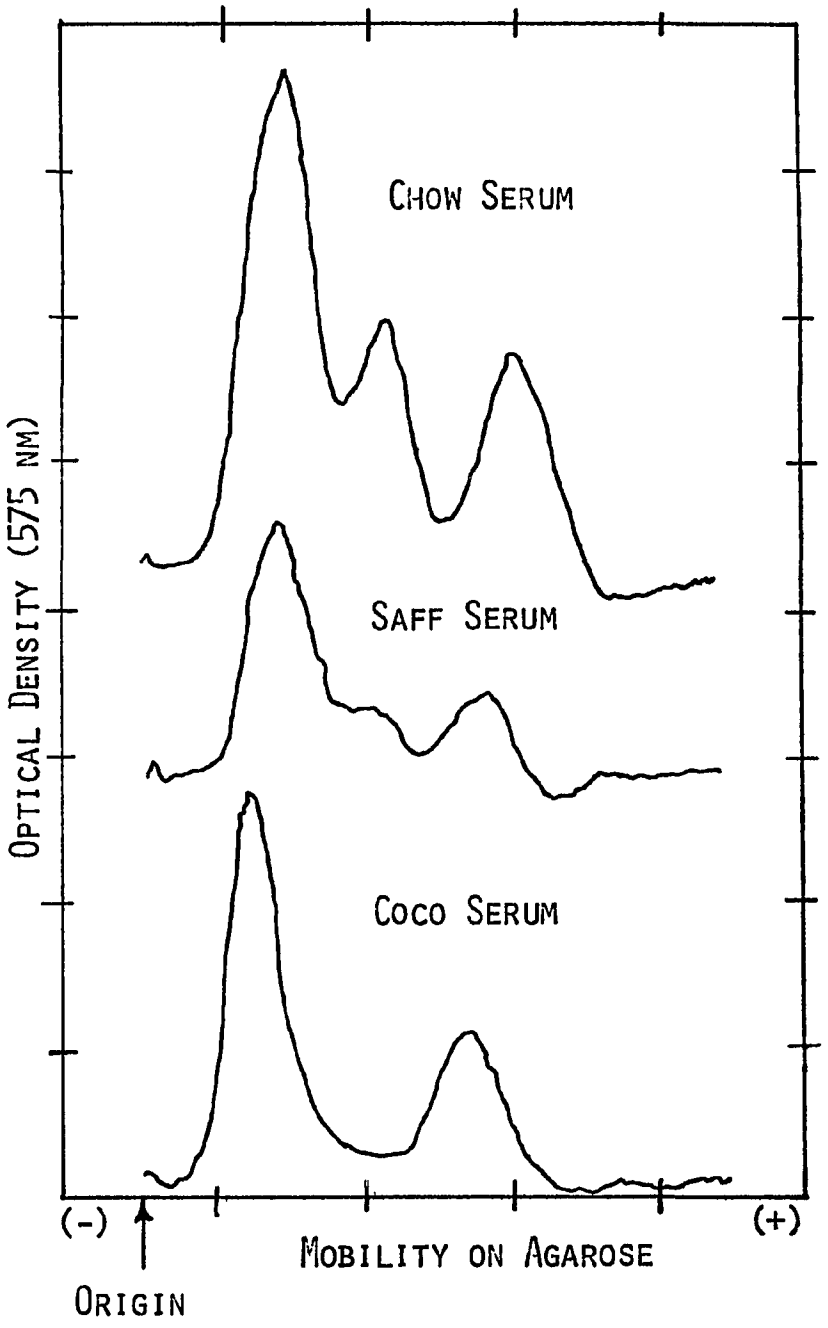

30
20
10
% OF PARTICLES
200
400
600
800
1000
DIAMETER (Å)
CHOW – VLDL

Figure 6. Densitometric scans of serum after agarose electrophoresis from rabbits fed the various diets. The gels were stained with fat red 7B and scans were performed at 575 nm.


CHOW SERUM
SAFF SERUM
COCO SERUM
OPTICAL DENSITY (575 NM)
(-)
(+)
ORIGIN
MOBILITY ON AGAROSE

absence of the pre-beta lipoprotein fraction normally present. Lipoproteins of pre-beta mobility on agarose gels correspond to the VLDL ($d < 1.006$ g/ml) from normal serum. Because of this finding, VLDL and LDL from rabbits after each dietary treatment were isolated and agarose gel electrophoresis was performed. Figures 7, 8 and 9 show the relative mobilities of the VLDL ($d < 1.006$ g/ml) and LDL ($1.019 < d < 1.063$ g/ml) isolated from rabbits fed the various diets. In the case of lipoproteins from rabbits fed either the chow or the SAFF diet (Figures 7 and 8) the $d < 1.006$ g/ml lipoprotein remained pre-beta in its mobility on agarose, and the 1.006 g/ml $< d < 1.063$ g/ml fraction had beta mobility. When agarose electrophoresis was performed on the isolated lipoproteins from the COCO diet-fed animals, however, both the VLDL and LDL were of β-mobility (Figure 9). This alteration in electrophoretic behavior points out that some change in the net charge of the VLDL particle has occurred (possibly in its protein moiety). Results similar to these found in the COCO diet case have been reported in the VLDL of rabbits made cholesterolemic via supplementary cholesterol feeding (78) indicating a possible similar relationship between the altered VLDL from these two dietary cases. VLDL which floats at $d < 1.006$ g/ml and is beta in its agarose mobility has been termed B-VLDL.

The possibility exists that there may be β-migrating VLDL (B-VLDL) in the serum of rabbits fed the SAFF diet as well as the normal pre-beta VLDL. Yet when the $d < 1.006$ g/ml fraction was isolated from serum of rabbits fed the SAFF diet and their electrophoretic mobilities determined, only one band, with normal pre-β mobility was seen. Consequently, the altered B-VLDL seen in animals fed the COCO diet must somehow be related to the high saturated fat content and not merely to the high total fat content of

Figure 7. Densitometric scans of serum VLDL and LDL after agarose electrophoresis from rabbits fed the chow diet indicating their relative mobilities (pre-beta and beta). Conditions were as described in Figure 6.

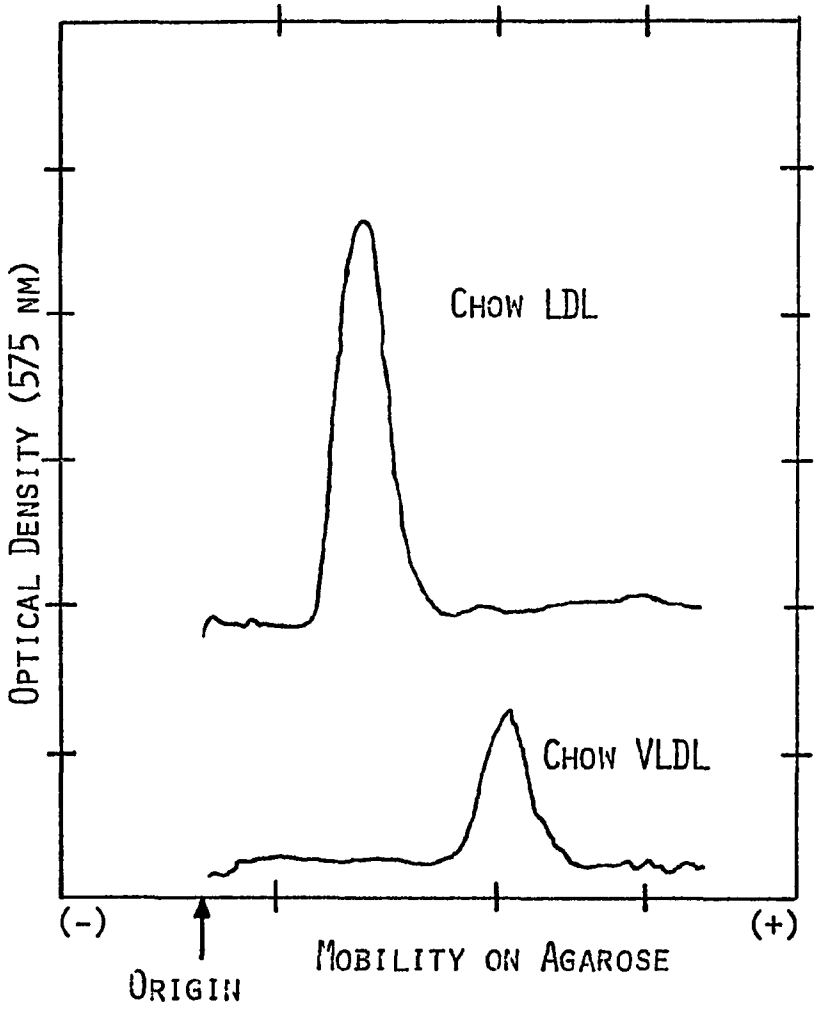

Optical Density (575 nm)
Chow LDL
Chow VLDL
(-)
(+)
Origin
Mobility on Agarose

Figure 8. Densitometric scans of serum VLDL and LDL after agarose electrophoresis from rabbits fed the SAFF diet indicating their relative mobilities (pre-beta and beta). Conditions were as described in Figure 6.

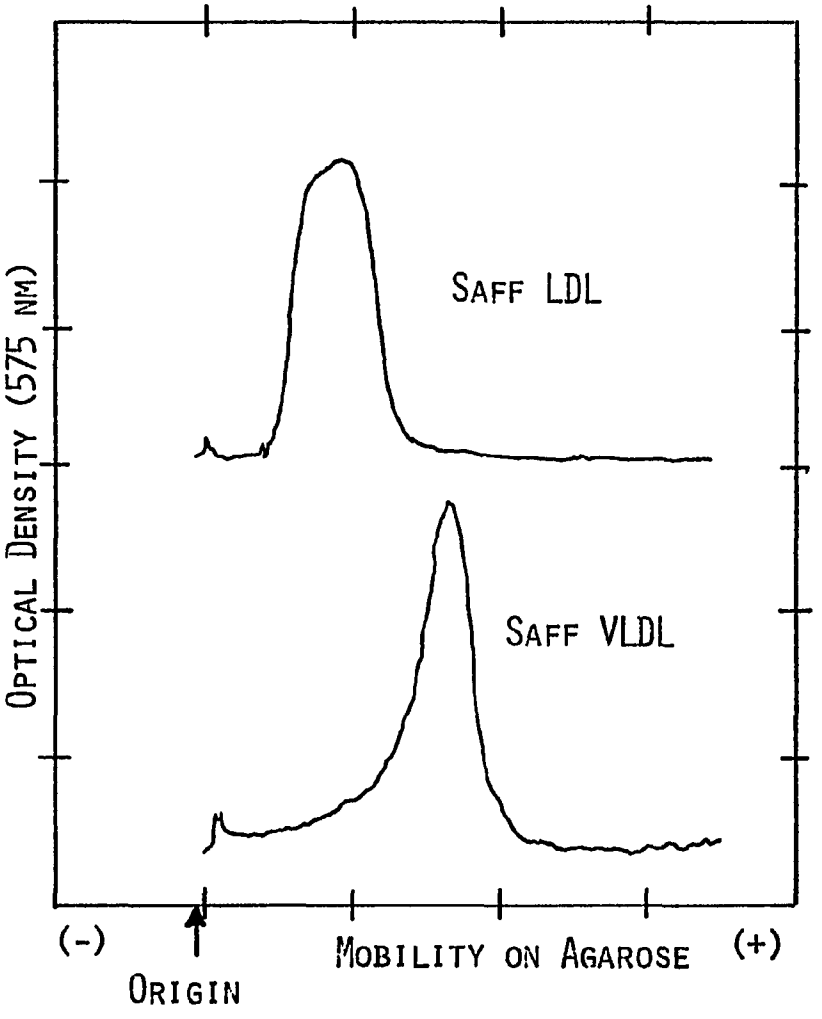
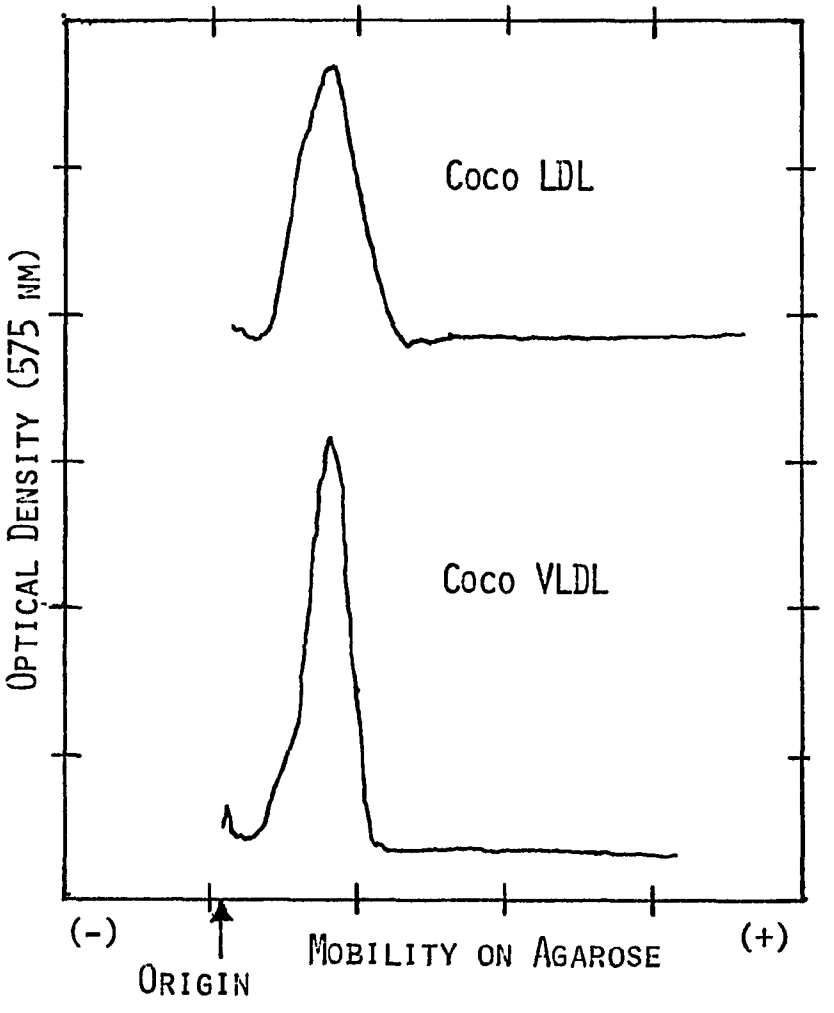

SAFF LDL
SAFF VLDL
OPTICAL DENSITY (575 NM)
(-)
ORIGIN
MOBILITY ON AGAROSE
(+)

Figure 9. Densitometric scans of serum VLDL and LDL after agarose electrophoresis from rabbits fed the COCO diet indicating their relative mobilities (both were beta). Conditions were as described in Figure 6.


Coco LDL
Coco VLDL
Optical Density (575 nm)
(-)
Origin
Mobility on Agarose
(+)

this diet. (The SAFF diet is as high in total fat as the COCO diet but highly polyunsaturated.)

The electrophoretic mobility of the LDL_1 (or IDL, $1.006 < d < 1.019$ g/ml) from rabbits fed either the SAFF or COCO diet were in the beta range with no distinct differences among the groups. HDL as well consistently migrated with α-mobility whether isolated from rabbits fed either of the dietary treatments employed.

F. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Rabbit Serum Apolipoproteins: Variation with Diet

Associated with the changes in agarose electrophoretic mobility and chemical composition of the rabbit VLDL was an alteration in the apolipoprotein composition among the dietary treatments. While changes in the mobility of apo LDL (apo B) and apo HDL (apo A) did not occur, one particular apo VLDL peptide was markedly increased with respect to the other apo VLDL peptides normally present. Figure 10 shows the densitometric scans of apo VLDL peptides after SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. Zone 1 of Figure 10 corresponds to the apo B peptide which is quite polymorphic while Zones 2 and 4 represent the apo C peptides. Zone 3 corresponds to the arginine-rich peptide (apo E) component of VLDL. These zones were assigned to include the respective peptides mentioned based on the typical behavior of rabbit apo VLDL in 14% SDS-polyacrylamide gels after comparisons of the mobilities of apo LDL, apo VLDL and apo HDL peptides on similar gels.

Table 6 shows the apolipoprotein composition of VLDL from rabbits fed the various diets. Chow-fed rabbit VLDL contained 60% apo B and 32% apo C peptides with only about 8% of the apolipoprotein made up of the

Figure 10. Densitometric scans of apo VLDL peptides after sodium dodecyl sulfate polyacrylamide gel electrophoresis from rabbits fed the various diets. The gels were stained with Coomassie blue and the scans were performed at 675 nm. The abbreviations used were as indicated in Figure 1. Zones 1, 2, 3 and 4 are described in the text, page 64.

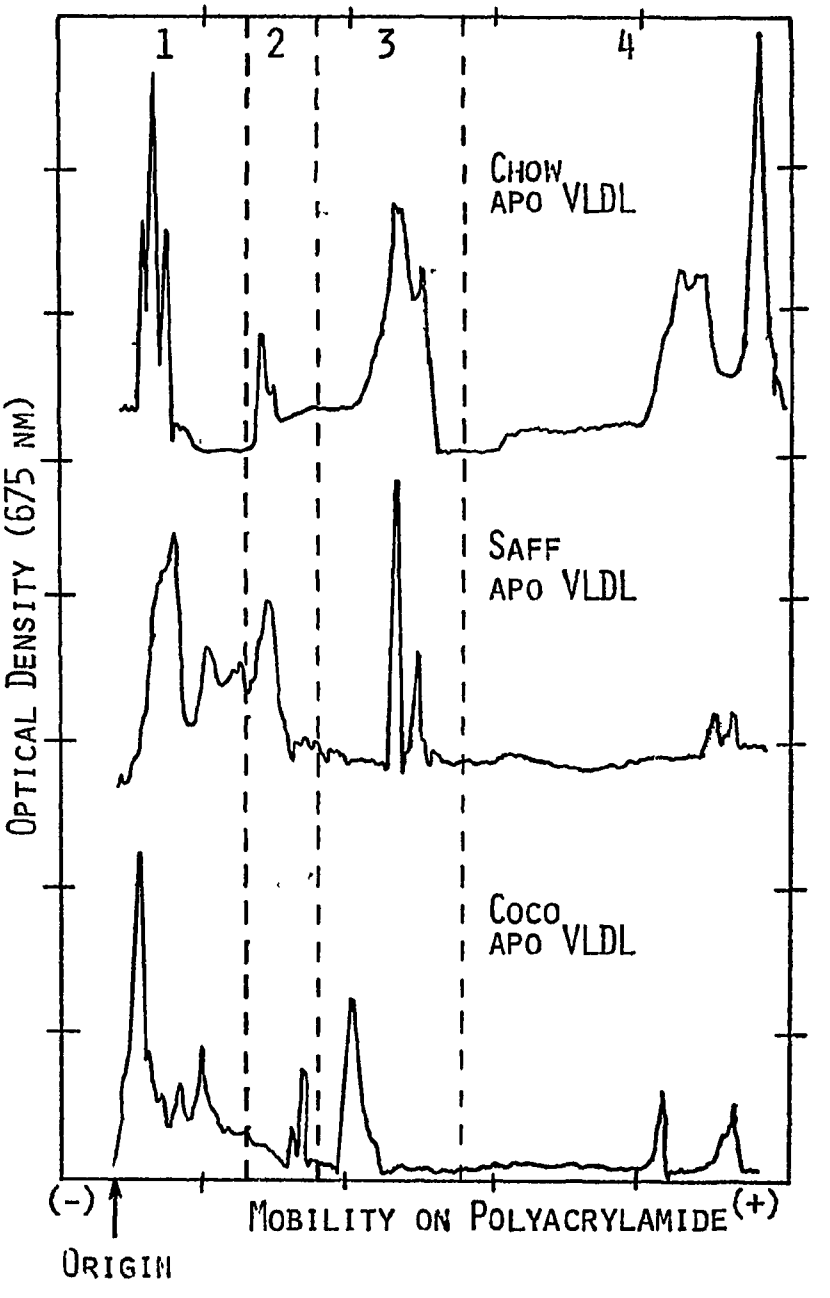

1
2
3
4
CHOW APO VLDL
SAFF APO VLDL
COCO APO VLDL
OPTICAL DENSITY (675 NM)
(-)
ORIGIN
MOBILITY ON POLYACRYLAMIDE
(+)

Table 6. Apo-VLDL % Composition of Rabbits on Different Diets

Diet	Zone 1 (Apo-B)	Zone 3 (Arg-Rich)	Zones 2 & 4 (Apo-C)
Chow	60 (1.1)*	8 (0.1)	32 (0.6)
SAFF	58 (1.9)	17 (0.5)	25 (0.8)
COCO	45 (3.3)	39 (2.8)	16 (1.2)

*Figures in parentheses represent mg VLDL protein/dl serum

arginine-rich peptide. Apo VLDL from rabbits fed the SAFF diet, while containing a similar relative amount of apo B (58%), was made up of 25% apo C peptide and more than twice as much of the arginine-rich peptide (17%) as the chow-fed rabbit VLDL. In the case of the COCO diet-fed rabbits, the serum VLDL apoproteins contained only 45% apo B while the apo C peptides decreased by half to 16%. Additionally the arginine-rich peptide accounted for nearly 40% of the total VLDL apoprotein composition, almost a five-fold increase, when compared to the chow case. Furthermore, increases in the absolute amounts of all apo peptides were noted in the case of the experimental diets. This finding is consistent with the increased amount of VLDL seen in rabbits fed the experimental diets. In summary, then, not only was there an increase in the relative and absolute amounts of the arginine-rich peptide in apo VLDL after feeding the COCO diet, but also a decrease in the contribution of the apo C peptides to the total apo VLDL. A similar finding was also observed, but to a lesser extent, after the feeding of the SAFF diet to the rabbits. This result may be responsible for the decreased mobility of the VLDL in agarose gels in that the increased amount of the arginine-rich peptide, a positively charged species at the pH employed, migrates less toward the anode during the application of the electrophoretic potential than normal VLDL, which is less positively charged.

G. Fatty Acid Composition of Lipid Classes of Rabbit Serum Lipoproteins: Variation with Diet

As expected, the serum lipoprotein fatty acid compositions reflect the type of dietary fat supplied to the rabbits. In the chow-fed case (Table 7), the fatty acids most prevalent in the diet (16:0, 18:1 and 18:2)

Table 7. Fatty Acid Composition (Mean % ± S.E.) of Lipid Classes of Plasma Lipoproteins in Rabbits Fed the Chow Diet (n = 5)

	Triglycerides			Phospholipids			Cholesteryl Esters		
Fatty Acid	VLDL	LDL	HDL	VLDL	LDL	HDL	VLDL	LDL	HDL
12:0	-	0.6±0.2	tr	0.6±0.2	0.6±0.1	0.5±0.1	-	0.9±0.3	tr
14:0	4.4±0.7	2.9±0.5	1.9±0.4	1.7±0.5	0.8±0.3	1.5±0.4	1.5±0.6	0.8±0.3	1.9±0.5
14:1	2.8±0.6	1.6±0.4	0.5±0.1	1.4±0.3	0.5±0.2	0.6±0.1	tr	1.1±0.4	tr
16:0	28.9±1.0	30.0±2.1	31.4±1.8	26.3±1.1	26.8±1.4	28.6±2.1	29.6±2.1	21.9±1.9	32.0±4.3
16:1	7.1±1.4	6.3±1.0	5.6±0.9	4.1±1.9	2.9±0.9	5.2±1.5	5.5±1.2	7.0±1.9	5.8±1.1
18:0	4.6±0.8	5.0±0.9	6.9±0.8	19.3±1.8*	21.8±1.9*	18.6±2.0*	4.8±1.0	5.2±0.9	7.1±1.9
18:1	30.1±3.4	32.0±3.1	32.0±3.0	19.0±1.1*	16.3±0.9*	19.3±1.3*	33.1±2.2	41.7±4.1	33.1±4.0
18:2	19.5±1.2	19.9±1.0	21.3±1.8	26.3±1.9	26.8±1.4	24.6±2.0	22.2±2.4	21.3±1.9	20.0±1.8
>18:2 <20:4	2.3±0.9	1.4±0.4	1.6±0.5	0.7±0.2	1.0±0.3	1.5±0.4	1.6±0.5	0.8±0.4	0.2±0.1
20:4	tr	tr	tr	0.6±0.2	2.4±0.6	tr	1.0±0.3	-	tr

*Substantially different from stearic acid content of chow diet fed

were also most prevalent in the triglyceride, phospholipid and cholesteryl ester of all lipoprotein fractions. The only exception was that nearly 20% of the phospholipid moiety of VLDL, LDL and HDL consisted of stearic acid (18:0) which may have resulted via chain elongation of shorter-chain saturated fatty acids, deacylation-reacylation or perhaps de novo synthesis of fatty acids. This increase in relative amount of stearic acid appeared to have been at the expense of oleic acid which is somewhat reduced when compared to the amount of oleic acid in the fat of the chow diet.

The fatty acid compositions of lipoprotein lipids after feeding the animals either the SAFF or COCO diet are shown in Tables 8, 9, 10 and 11. The large difference in the saturation level of these two diets was reflected in the fatty acid composition of the lipoprotein lipids (triglycerides, cholesteryl esters, and phospholipids). The greatest differences were found in the palmitate (16:0), oleate (18:1) and linoleate (18:2) content of the three lipid classes. In general, the percentages of palmitate and oleate were significantly decreased during feeding of the polyunsaturated fat diet (SAFF diet) while linoleate was consistently increased in each lipid class after the feeding of this diet when compared to the COCO case. Also the fatty acid composition of the lipoprotein triglycerides and cholesteryl esters were most affected by SAFF diet feeding while the phospholipid class showed the least change. One exception, however, is that in the case of IDL lipids, an increase rather than a decrease of oleic acid was observed (Table 9) after feeding the SAFF diet.

Table 8. Fatty Acid Analysis of Rabbit VLDL Lipids after SAFF or COCO Diet Feeding

(Mean % ± S.E.) (n = 5)

	Triglycerides		Phospholipids		Cholesteryl Esters	
Fatty Acid	SAFF	COCO	SAFF	COCO	SAFF	COCO
12:0	-	-	-	-	-	1.0±0.4
14:0	-	17.7±1.2	-	3.0±0.9	-	5.6±0.9
14:1	-	-	-	-	-	-
16:0	27.3±1.0[c]	38.5±3.0[c]	24.2±1.4	24.1±1.3	15.8±1.4[b]	25.8±1.8[b]
16:1	1.5±1.0	6.5±1.3	4.7±0.6	1.3±0.5	6.0±1.0	6.2±0.9
18:0	13.2±1.5	11.8±1.5	19.2±1.9	20.3±2.1	4.7±0.8	11.1±1.1
18:1	17.5±1.6	24.4±3.1	14.2±1.3[b]	26.5±1.8[b]	32.5±3.2	42.5±2.9
18:2	40.4±3.2[a]	1.2±0.2[a]	37.8±1.1[b]	24.8±1.3[b]	41.0±3.4[a]	7.9±1.1[a]
>18:2 <20:4	tr	-	-	-	tr	-
20:4	tr	tr	-	tr	tr	tr

[a]Significantly different at $p < .001$ level

[b]Significantly different at $p < .01$ level

[c]Significantly different at $p < .02$ level

Table 9. Fatty Acid Analysis of Rabbit IDL Lipids after SAFF or COCO Diet Feeding (Mean % ± S.E.) (n = 5)

	Triglycerides		Phospholipids		Cholesteryl Esters	
Fatty Acid	SAFF	COCO	SAFF	COCO	SAFF	COCO
12:0	-	-	-	1.7±0.6	-	-
14:0	-	23.3±1.0	-	4.1±0.9	-	2.6±1.0
14:1	-	-	-	-	-	-
16:0	34.0±2.8	35.8±2.8	23.7±1.9	25.9±2.1	20.1±1.9[b]	29.4±1.8[b]
16:1	tr	4.4±0.9	1.9±0.9	1.8±0.5	4.5±0.9	3.6±0.8
18:0	11.3±1.2[b]	18.1±1.8[b]	6.5±1.0[a]	27.7±1.7[a]	4.8±0.9	12.4±1.0
18:1	34.7±2.1[b]	16.6±1.6[b]	47.4±1.9[a]	21.2±2.0[c]	50.3±2.0[c]	41.8±2.4[c]
18:2	19.3±1.8[a]	1.8±0.8[a]	20.4±1.7	17.7±2.1	20.4±1.9[b]	10.1±1.8[b]
>18:2 <20:4	-	-	-	-	-	tr
20:4	0.7±0.2	tr	tr	tr	tr	tr

[a]Significantly different at $p < .001$ level

[b]Significantly different at $p < .01$ level

[c]Significantly different at $p < .05$ level

tr refers to trace amount (<0.5%)

Table 10. Fatty Acid Analysis of Rabbit LDL Lipids after SAFF or COCO Diet Feeding (Mean % ± S.E.) (n = 5)

	Triglycerides		Phospholipids		Cholesteryl Esters	
Fatty Acid	SAFF	COCO	SAFF	COCO	SAFF	COCO
12:0	-	-	-	tr	-	-
14:0	-	22.3±2.1	-	2.9±0.9	-	5.1±1.1
14:1	-	-	-	-	-	-
16:0	28.8±1.1[d]	37.4±1.9[d]	32.0±2.1	26.2±1.9	13.2±1.8[b]	28.4±2.5[b]
16:1	tr	4.5±0.9	tr	1.7±0.5	2.4±0.8	2.4±0.8
18:0	6.9±1.0[c]	13.5±1.2[c]	29.6±1.9	30.2±2.2	2.7±0.9[c]	8.7±1.2[c]
18:1	27.9±1.8	20.6±1.8	5.4±1.0[b]	21.7±2.0[b]	34.0±1.9[c]	46.3±2.8[c]
18:2	34.8±2.4[a]	1.8±0.5[a]	32.0±3.0[b]	17.4±1.9[b]	47.7±3.4[a]	9.2±1.3[a]
>18:2 <20:4	-	-	-	-	-	-
20:4	1.6±0.6	-	1.0±0.6	-	-	tr

[a]Significantly different at p < .001 level

[b]Significantly different at p < .01 level

[c]Significantly different at p < .02 level

[d]Significantly different at p < .05 level

tr refers to trace amount (0.5%)

Table 11. Fatty Acid Analysis of Rabbit HDL Lipids after SAFF or COCO Diet Feeding (Mean % ± S.E.) (n = 5)

	Triglycerides		Phospholipids		Cholesteryl Esters	
Fatty Acid	SAFF	COCO	SAFF	COCO	SAFF	COCO
12:0	-	5.5±1.0	-	tr	-	tr
14:0	-	16.6±1.6	-	2.0±0.8	-	6.4±0.9
14:1	-	-	-	tr	-	-
16:0	29.1±2.1	30.7±2.3	31.0±2.6	22.6±4.1	20.7±1.8	23.3±1.8
16:1	tr	4.2±1.0	1.2±0.9	1.4±0.8	tr	6.6±0.9
18:0	7.2±1.1[c]	13.4±2.0[c]	28.5±1.2	29.9±1.0	2.5±0.7	8.9±1.3
18:1	26.2±1.9	24.3±2.2	6.9±1.1[b]	21.5±2.1[b]	38.5±2.1	39.5±2.4
18:2	35.1±2.2[a]	5.4±1.1[a]	31.6±2.4	21.1±1.3	36.6±2.6[b]	15.4±1.9[b]
>18:2 <20:4	-	-	-	tr	-	-
20:4	1.5±0.8	-	0.8±0.5	1.0±0.6	1.6±0.7	-

[a]Significantly different at $p < .001$ level

[b]Significantly different at $p < .01$ level

[c]Significantly different at $p < .02$ level

tr refers to trace amount (<0.5%)

Section II

A. Characterization of the Triacylglycerol Hydrolase Activities of Enzyme Preparations

Two separate preparations of triglyceride hydrolase activities from chow-fed animals were characterized. Activities from similar preparations from animals fed the experimental diets were measured as well. Both artificial triglyceride emulsions and natural (VLDL) substrates were used. The two activities characterized consisted of partially purified lipases obtained from post-heparin plasma of either intact rabbits (hereafter referred to as PHP LPL) or supradiaphragmatic animals (EHP LPL) after the injection of sodium heparin (see Materials and Methods).

1. Enzyme Activities Using VLDL as Substrate: Variation of Substrate with Diet

The triacylglycerol hydrolase activities of both the PHP LPL and EHP LPL preparations (d > 1.063 g/ml) were determined using radiolabeled VLDL as substrate. Table 12 shows the post-heparin lipase (d > 1.063 g/ml) activities of enzyme preparations from rabbits fed the chow diets using VLDL from animals fed either the chow or the experimental diets as substrate. These activities were determined in order to test whether the VLDL isolated from rabbits fed the experimental diets showed any appreciable differences as substrate for the enzyme preparations used. As seen in Table 12, the SAFF diet-fed animals' VLDL may be a slightly better substrate than the COCO diet rabbit VLDL for both the EHP LPL and PHP LPL activities, yet this difference is probably not highly significant. When VLDL from chow-fed animals is used as substrate, lipase activities were appreciably higher in each case.

Table 12. Triacylglycerol Hydrolase Activities of d > 1.063 g/cm^3 Post-heparin Plasma Fractions of Chow-fed Animals Against Natural Substrates (VLDL) from Rabbits Fed Either the COCO, SAFF, or Chow Diet (Average of Two Determinations)

Substrate-Enzyme Preparation	Lipase Activity	
	μmoles FFA released ml/hr	nmoles FFA released ml/min
SAFF-VLDL - EHP LPL	7.9	131
SAFF-VLDL - PHP LPL	10.9	182
COCO-VLDL - EHP LPL	6.8	114
COCO-VLDL - PHP LPL	9.5	159
Chow VLDL - EHP LPL	11.2	187
Chow VLDL - PHP LPL	15.1	252

2. Characteristics of Enzyme Activities Using Artificial Substrates

Table 13 contains the post-heparin plasma triacylglycerol hydrolase activities from both intact chow-fed rabbits (PHP LPL) and supradiaphragmatic chow-fed rabbits (EHP LPL). The enzyme activities of both preparations increased nearly two times after the d < 1.063 g/ml lipoproteins were removed. Consequently all further experiments were conducted after removing this fraction of lipoprotein. The activities of these enzyme preparations were further characterized according to parameters typically used for lipoprotein lipase. These results follow.

a. PHP LPL Activity

Substrate concentration was not rate limiting at about 6.2 mM triolein in an egg lecithin stabilized emulsion (Figure 11). Under standardized assay conditions (see Materials and Methods), the enzyme activity was linear with respect to time for at least 60 minutes (Figure 12). After this time the activity then decreased presumably due to the inactivation of the enzyme at the 37°C incubation temperature employed (109). Linearity of the activity was also noted with increasing amounts of enzyme (Figure 13). A reduction in the reaction rate was noted, however, when about 8-10% of the triolein substrate had been hydrolyzed. Lastly, the reaction showed a pH optimum of about 8.4 (Figure 14).

b. EHP LPL Activity

The lecithin stabilized triolein emulsion substrate became rate limiting at about 6.2 mM triolein (Figure 15). The EHP LPL activity was linear with respect to time for at least 60 minutes (Figure 16), and with respect to enzyme concentration (Figure 17) until the substrate became rate limiting.

Table 13. Partial Purification of the Triacylglycerol Hydrolase Enzyme from Post-heparin Plasma from Intact and Supradiaphragmatic Chow-fed Rabbits (Average of Three Determinations)

Purification Step	Lipase Activity: nmoles FFA released / ml/min	Lipase Activity: nmoles FFA released / mg protein/min	Protein: mg / ml	Purification
PHP LPL (d = 1.006)	47.3	0.85	55.5	
PHP LPL (d > 1.063)	207.0	1.39	148.0	1.6 x
EHP LPL (d = 1.006)	56.0	1.06	53.0	
EHP LPL (d > 1.063)	159.4	1.76	90.5	1.7 x

Figure 11. Effect of substrate concentration on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from intact rabbits (PHP LPL) dialyzed against sodium barbital buffer. Enzyme preparations in the amount of 0.04 ml were mixed with 0.09 ml of substrate containing the following final concentrations: labeled and unlabeled triolein, 0.6-7.9 μmoles/ml; lecithin, 0.51 μmoles/ml; albumin, 1% (w/v), and serum, 9% (v/v), in 5 mM sodium barbital buffer, pH 7.4 in saline. Final assay volume was 0.2 ml and incubations were carried out for 1 hour at 37°C.

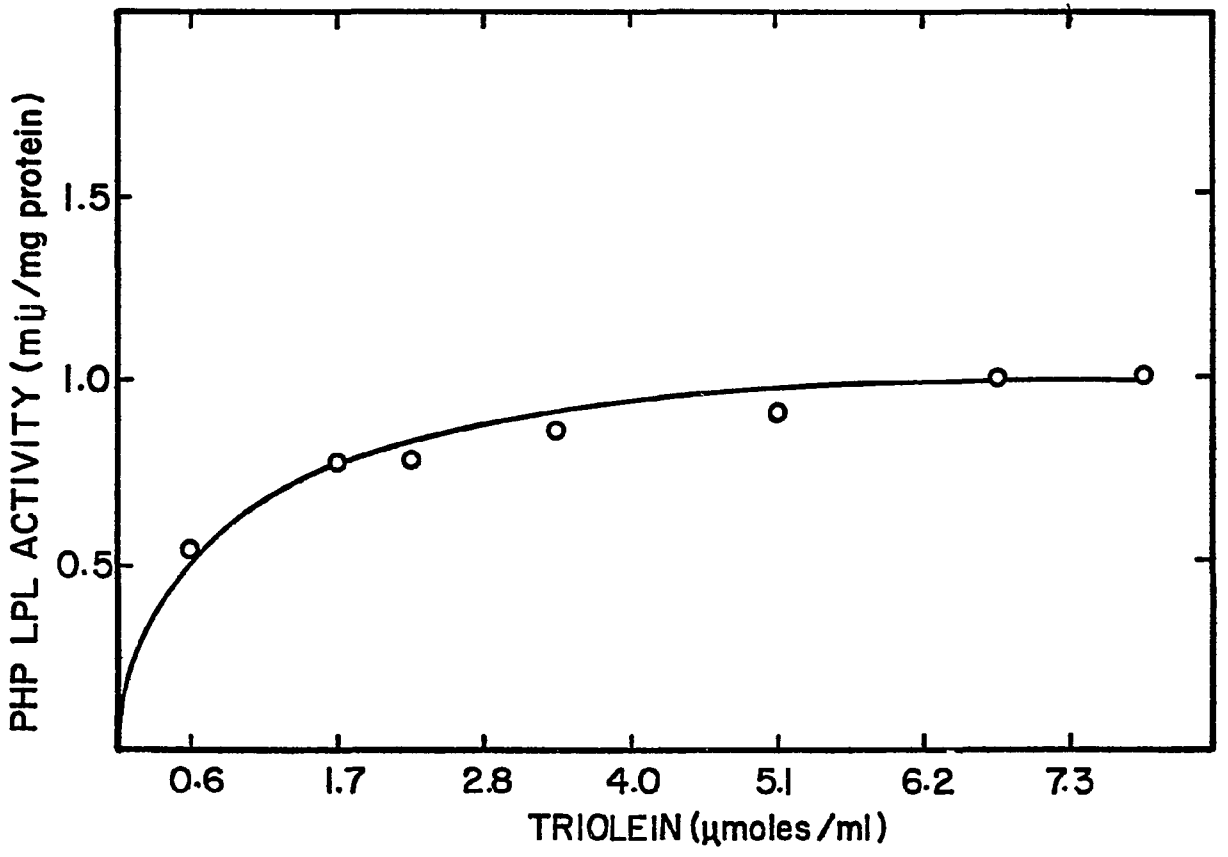

PHP LPL ACTIVITY (mμ/mg protein)
1.5
1.0
0.5
0.6
1.7
2.8
4.0
5.1
6.2
7.3
TRIOLEIN (μmoles/ml)

Figure 12. Effect of incubation time on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from intact rabbits (PHP LPL) dialyzed against sodium barbital buffer. Except for the variation of time of incubation and the use of 6.1 μmoles/ml final triolein concentration, all conditions were as described in Figure 11.

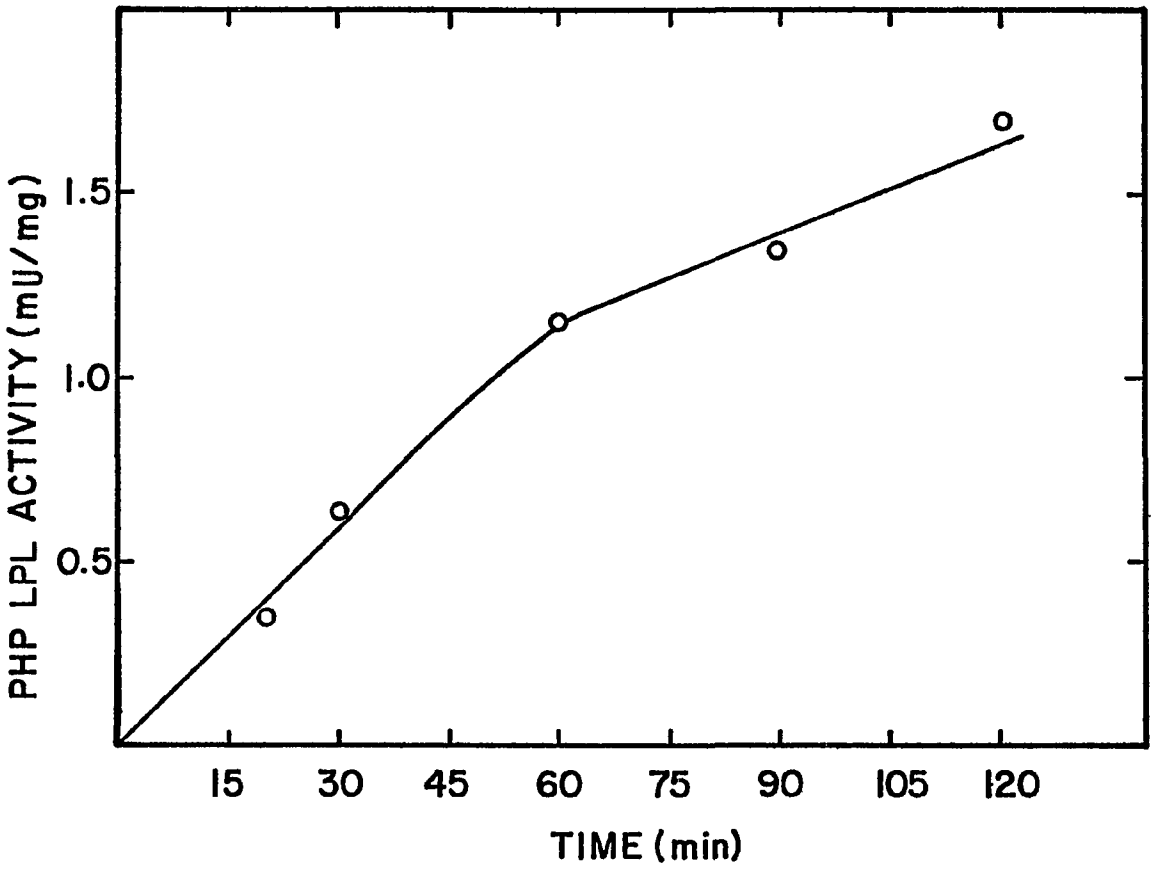

PHP LPL ACTIVITY (mU/mg)
1.5
1.0
0.5
15
30
45
60
75
90
105
120
TIME (min)

Figure 13. Effect of enzyme concentration (mg) on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from intact rabbits (PHP LPL) dialyzed against sodium barbital buffer. Appropriate volumes of the enzyme preparations containing 0.5-5.0 mg protein were incubated with substrate containing 6.1 µmoles triolein/ml in a final assay volume of 0.2 ml. All other conditions employed were as described in Figure 11.

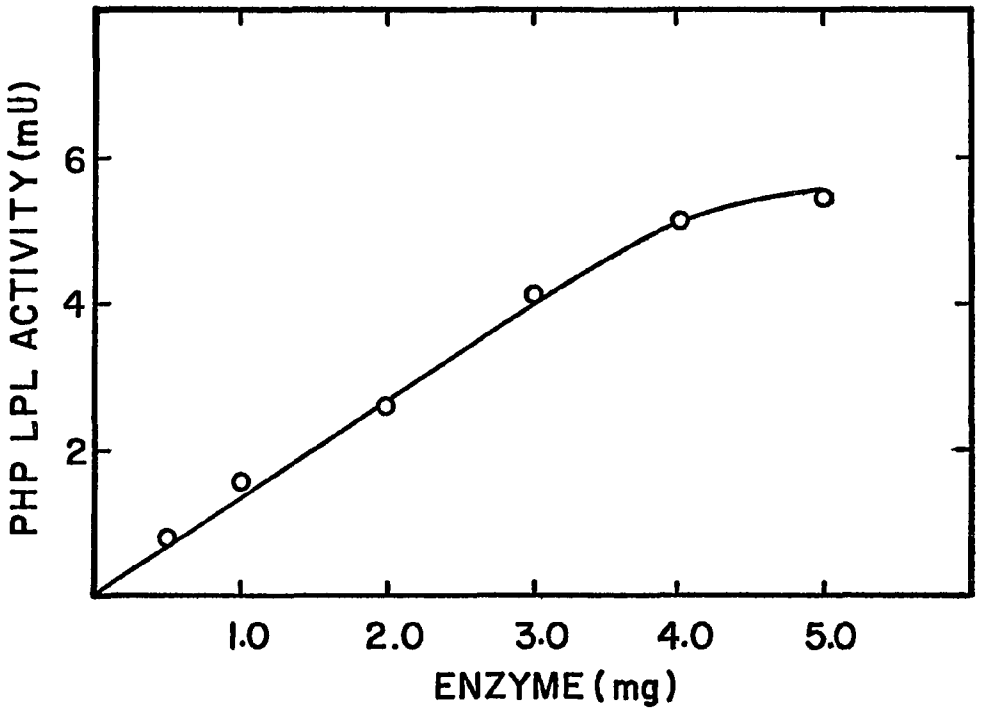

PHP LPL ACTIVITY (mU)
6
4
2
1.0
2.0
3.0
4.0
5.0
ENZYME (mg)

Figure 14. Effect of pH on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from intact rabbits (PHP LPL) dialyzed against sodium barbital buffer. While the pH of the substrate preparations and the buffer used was varied between 6.8 and 9.2, all other conditions were as described in Figure 11.

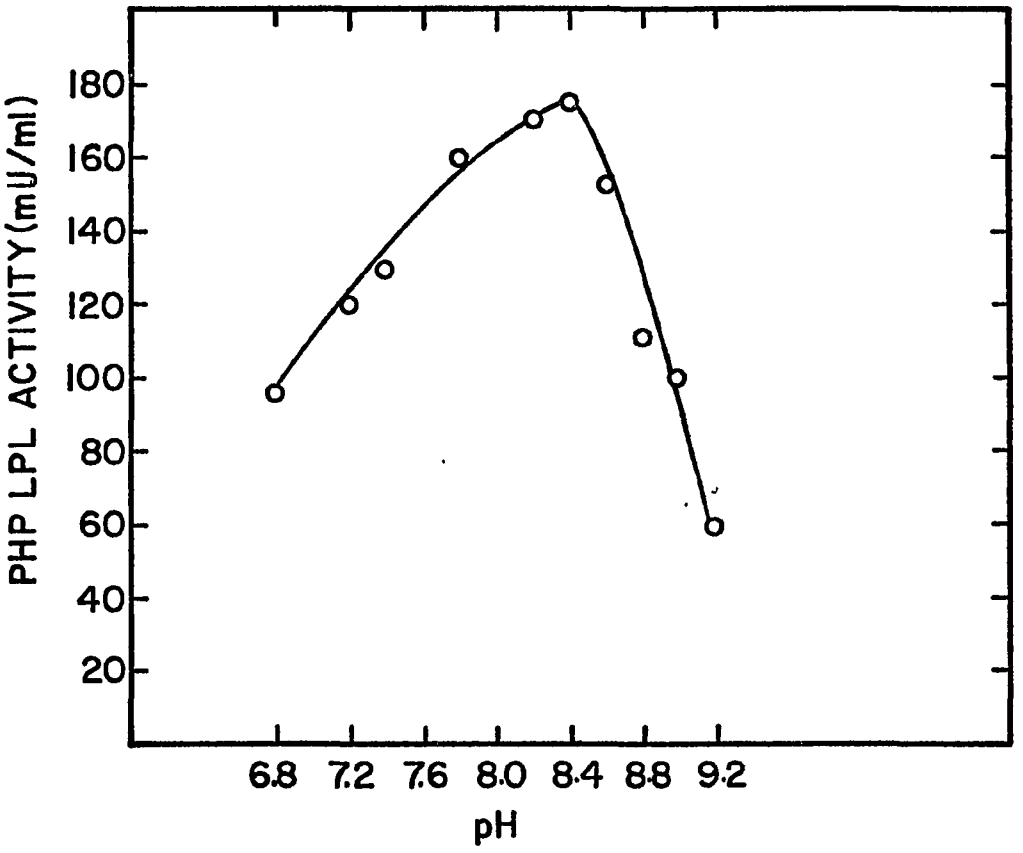

PHP LPL ACTIVITY (mU/ml)
180
160
140
120
100
80
60
40
20
6.8
7.2
7.6
8.0
8.4
8.8
9.2
pH

Figure 15. Effect of substrate concentration on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from supradiaphragmatic rabbits (EHP LPL) dialyzed against sodium barbital buffer. All assay conditions were as described in Figure 11.

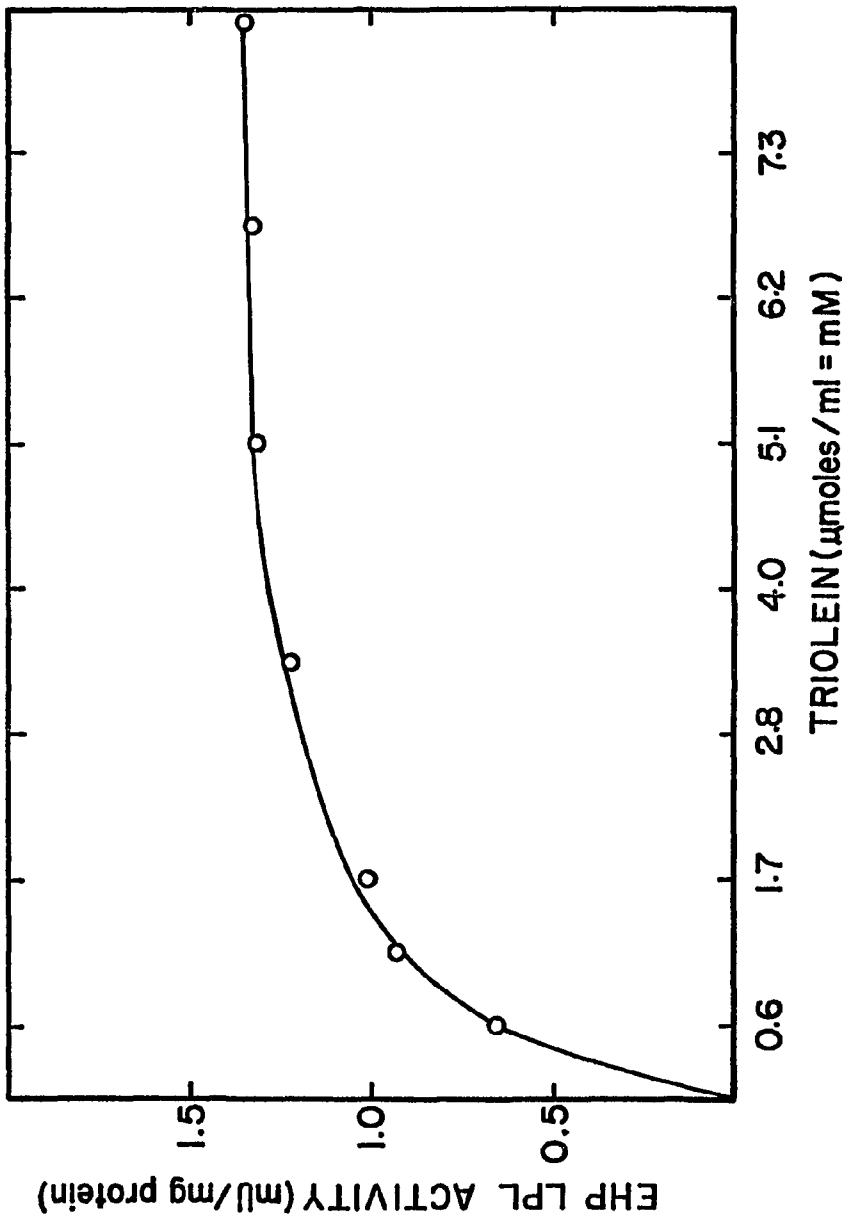

EHP LPL ACTIVITY (mU/mg protein)
0.5
1.0
1.5
0.6
1.7
2.8
4.0
5.1
6.2
7.3
TRIOLEIN (μmoles/ml = mM)

Figure 16. Effect of incubation time on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from supradiaphragmatic rabbits (EHP LPL) dialyzed against sodium barbital buffer. All assay conditions were as described in Figure 12.

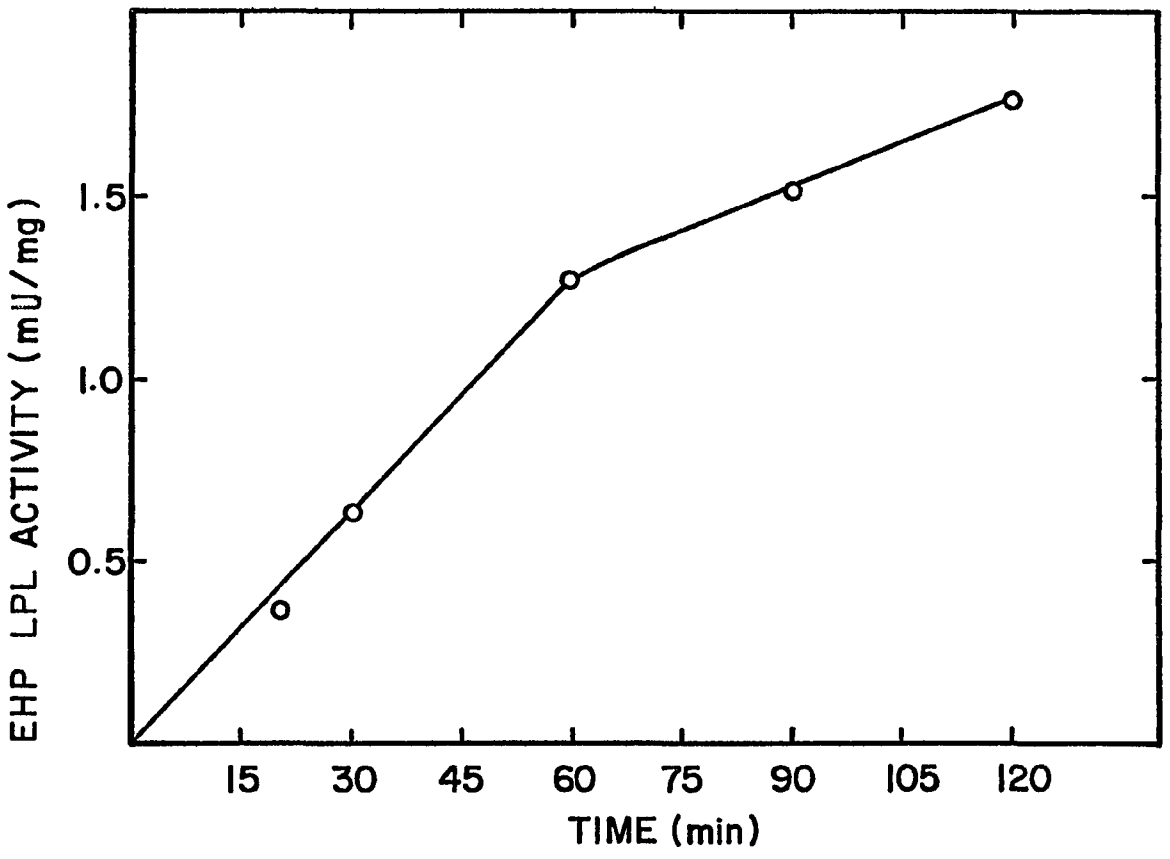
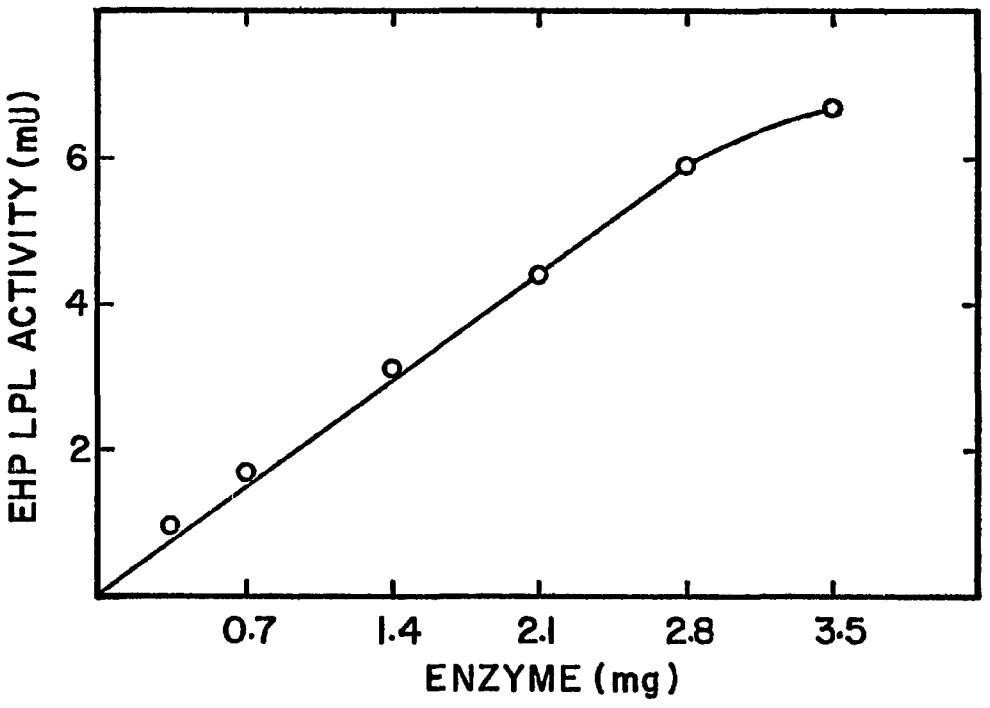

EHP LPL ACTIVITY (mU/mg)
1.5
1.0
0.5
15
30
45
60
75
90
105
120
TIME (min)

Figure 17. Effect of enzyme concentration (mg) on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from supradiaphragmatic rabbits (EHP LPL) dialyzed against sodium barbital buffer. Appropriate volumes of the enzyme preparations containing 0.35-3.5 mg protein were incubated with substrate containing 6.1 μmoles triolein/ml in a final assay volume of 0.2 ml. All other conditions employed were as described in Figure 11.


EHP LPL ACTIVITY (mU)
2
4
6
0.7
1.4
2.1
2.8
3.5
ENZYME (mg)

Again, a reduction in the rate of reaction was noted when about 8-10% of the triolein had been hydrolyzed. The pH optimum for this enzyme preparation was 8.4 as well (Figure 18).

Assay conditions for subsequent experiments employing either of the enzyme preparations were chosen based on the above mentioned results. These conditions have been described in detail elsewhere (see Materials and Methods), but, unless otherwise stated, reactions were allowed to run for 60 minutes at physiological pH and 0.15 M NaCl with enzyme and substrate concentrations such that no more than 10% of the triolein was hydrolyzed.

B. Effect of NaCl on the Triacylglycerol Hydrolase Activities from Different Sources

The effect of pre-incubation with NaCl on the triacylglycerol hydrolase activity from different sources can be seen in Figure 19. The enzyme preparations were pre-incubated at 27°C for 60 minutes in the presence of increasing amounts of NaCl, then assayed for lipase activity after adjusting the NaCl concentration to 0.15 M. As can be seen in the figure, the EHP LPL activity is inhibited approximately 96% at a preincubation NaCl concentration of 1.0 M. This finding is consistent with the effect of 1.0 M NaCl on the lipoprotein lipase enzyme originating in extra-hepatic or peripheral tissues (e.g. adipose tissue, cardiac muscle). The PHP LPL activity, after preincubating it with 1.0 M NaCl, is inhibited about 53% and shows that this preparation contains both hepatic and extra-hepatic triacylglycerol lipase activities. The hepatic triacylglycerol lipase activity is more resistant to higher NaCl concentrations (53) than the peripheral form. Thus the PHP LPL preparation, which contains both forms of lipase activity, is not completely inhibited. As the NaCl concentration

Figure 18. Effect of pH on the post-heparin plasma triglyceride lipase activity (d > 1.063 g/ml fraction) from supradiaphragmatic rabbits (EHP LPL) dialyzed against sodium barbital buffer. All assay conditions used were as described in Figure 14.

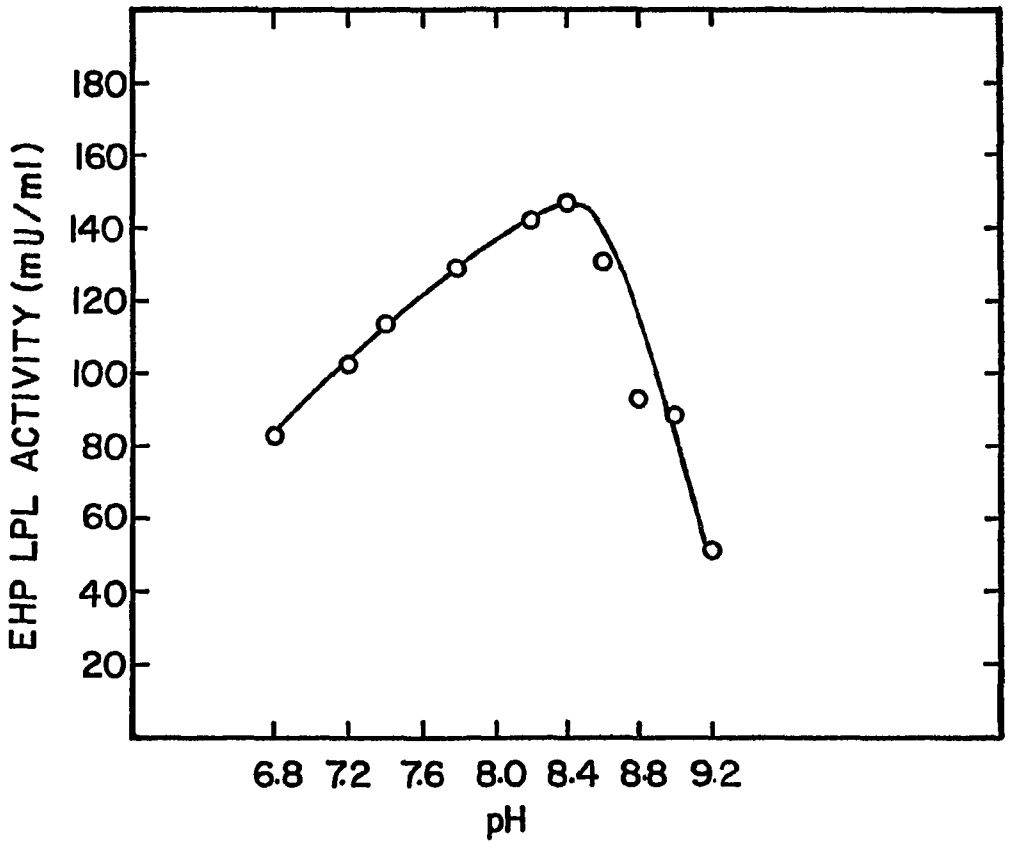
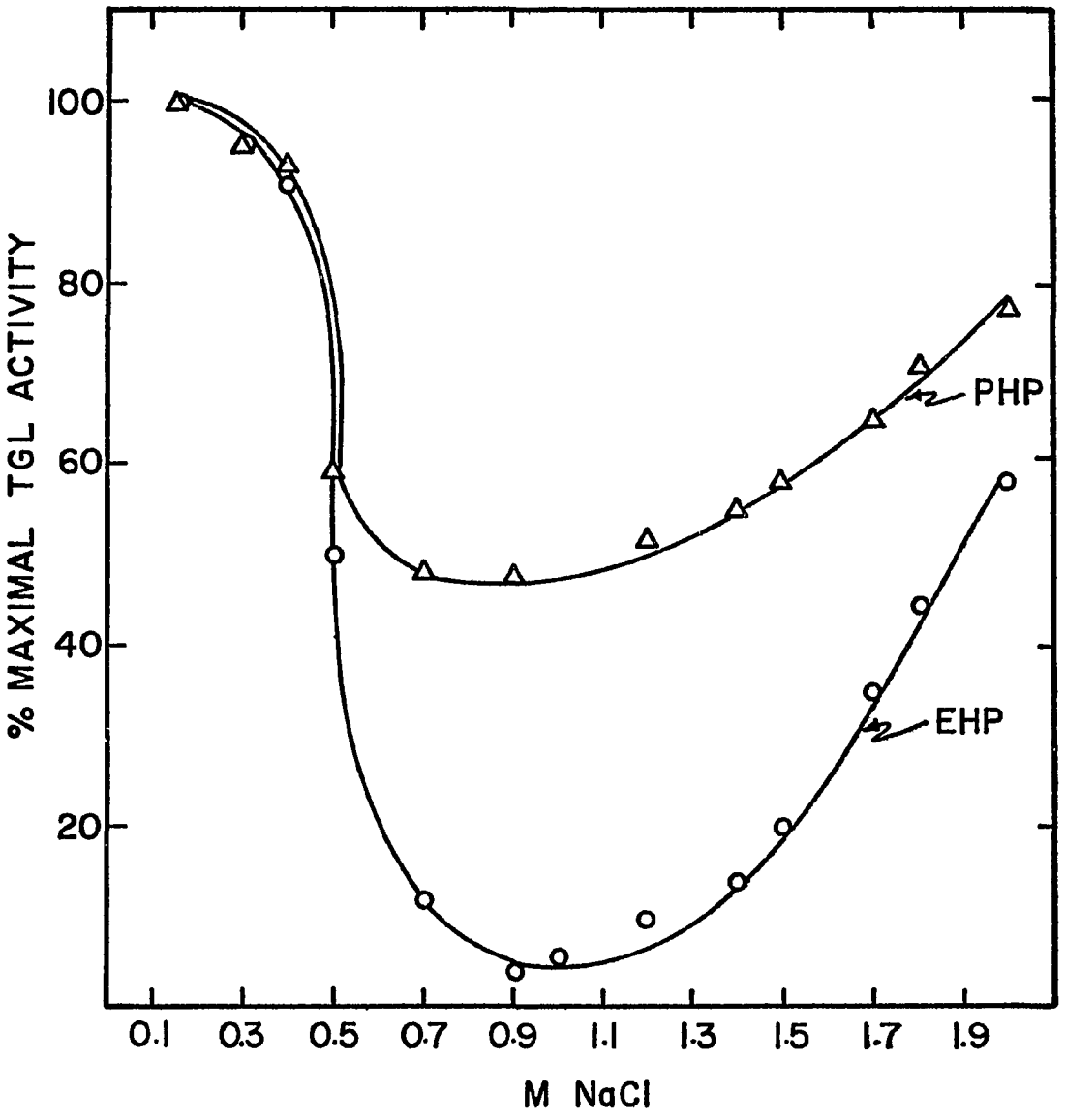

EHP LPL ACTIVITY (mU/ml)
180
160
140
120
100
80
60
40
20
6.8
7.2
7.6
8.0
8.4
8.8
9.2
pH

Figure 19. Effect of NaCl concentration on the post-heparin plasma triglyceride lipase activities (d > 1.063 g/ml fraction) from both intact and supradiaphragmatic rabbits (PHP LPL and EHP LPL, respectively) dialyzed against sodium barbital buffer. The equivalent of 0.01 ml of enzyme preparation was used in a total assay volume of 0.2 ml after preincubation of 0.04 ml of enzyme preparation with from 0.1-1.9 M NaCl for 60 minutes at 27°C. The assay was then conducted after adjusting the NaCl concentration to 0.15 M with barbital buffer at 37°C for 1 hour. The substrate mixture used was the same as that described in Figure 11 with 6.1 μmoles triolein/ml.


% MAXIMAL TGL ACTIVITY
100
80
60
40
20
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
1.9
M NaCl
PHP
EHP

is increased past about 1.3 M the lipase activities of both EHP LPL and PHP LPL preparations rose progressively. Higher salt concentrations, therefore, appear to exert some protective effect on the lipase activity. This effect has been previously described for lipoprotein lipase but remains unexplained (53).

C. The Selective Measurement of Peripheral and Hepatic Triacylglycerol Hydrolase Activities: Variation of Relative Activity with Diet

The effect of protamine sulfate concentration on the inhibition of triacylglycerol hydrolase activities from the two different sources is depicted in Figure 20. Under the assay conditions employed (see caption to Figure 20), it was found that prior incubation of enzyme with from 0.4-1.6 mg protamine/ml final assay produced maximal preservation of the PHP LPL activity with simultaneous maximal inactivation of the EHP LPL activity. For this reason PHP LPL and EHP LPL preparations ($d > 1.063$ g/ml) from chow, SAFF, and COCO diet-fed rabbits were assayed after preincubation of them with 1.0 mg of protamine/ml final assay or 5 mM barbital buffer for 10 minutes at 27°C in order to selectively measure the contributions of hepatic and peripheral forms of lipase in the PHP preparations of animals on the various diets. The results of this experiment can be seen in Table 14. Since it is impossible by this method to inactivate the peripheral form of the enzyme completely without some reduction of the hepatic form, a correction must be made.[1] The important trend to note in this table is that in the normal rabbit, more than half (57.1%) of the total lipase activity is due to

---

[1]Protamine-resistant TGLA = 0.1 (extra-hepatic TGLA) + 0.9(hepatic TGLA); protamine-inactivated TGLA = 0.9(extra-hepatic TGLA) = 0.1(hepatic TGLA). Thus, hepatic TGLA = 1.125(protamine-resistant TGLA) - 0.125(protamine-inactivated TGLA); and, extra-hepatic TGLA = 1.125(protamine-inactivated TGLA) - 0.125(protamine-resistant TGLA).

Figure 20. Effect of preincubation with increasing amounts of protamine sulfate on the inhibition of triglyceride lipase activities (d > 1.063 g/ml fraction) from both intact and supradiaphragmatic rabbits (PHP LPL and EHP LPL respectively) dialyzed against sodium barbital buffer. The equivalent of 0.01 ml of enzyme preparation was used in a total assay volume of 0.2 ml after preincubation of 0.04 ml of enzyme preparation with 0.2-2.0 mg protamine/ml final assay for 10 minutes at 27°C. The assay was then conducted as described in Figure 11 with a triolein concentration of 6.1 µmoles/ml.

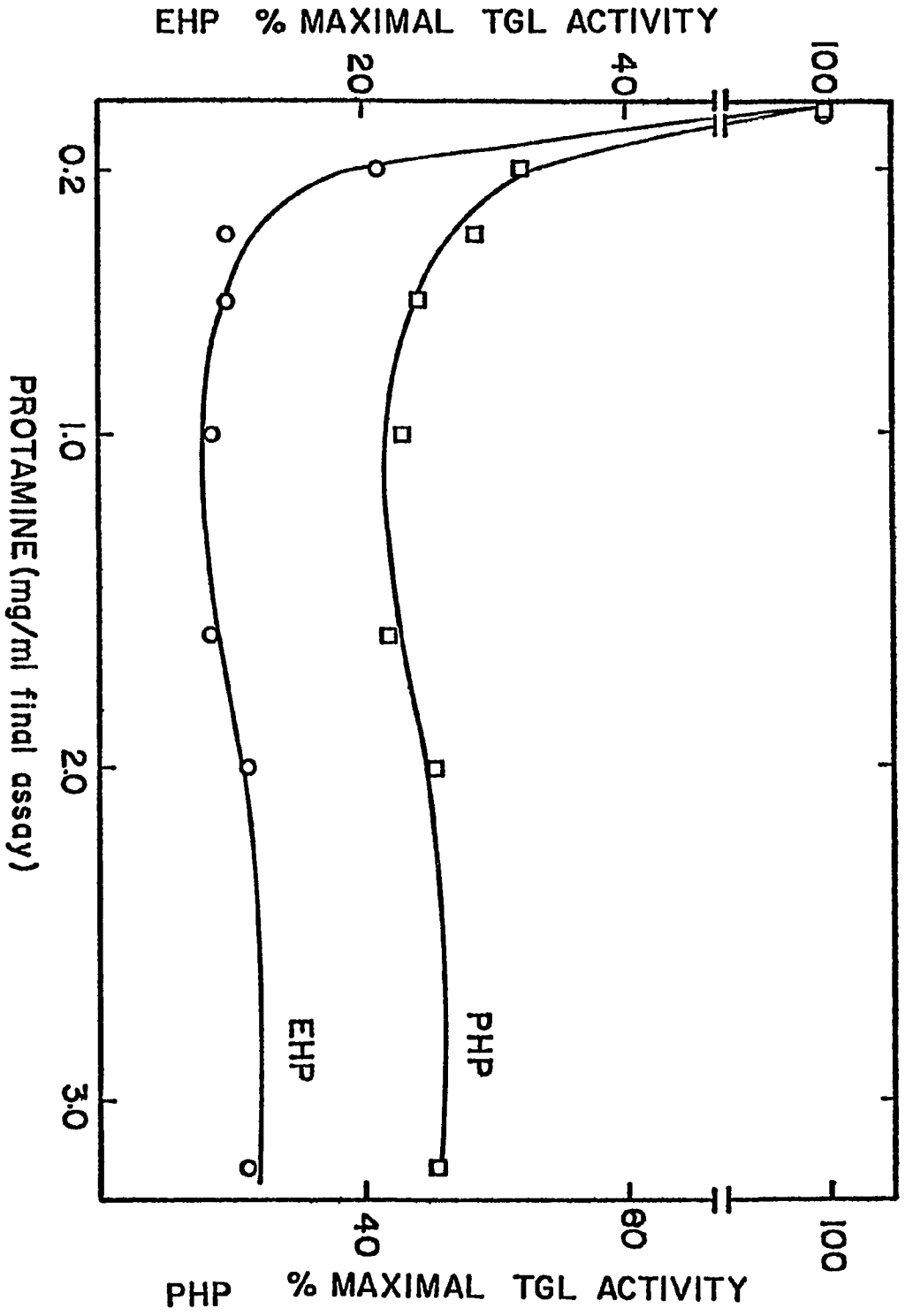

EHP % MAXIMAL TGL ACTIVITY
20
40
100
0.2
1.0
2.0
3.0
PROTAMINE (mg/ml final assay)
EHP
PHP
40
60
100
PHP % MAXIMAL TGL ACTIVITY

Table 14. Contributions of Hepatic and Extra-hepatic Triacylglycerol Hydrolase Activities to Total Post-heparin Plasma Lipase Activity[a] (Average of Three Determinations)

		TGL Activity (mU/ml)			
Dietary Group	Total PHP LPL	Protamine Resistant	Protamine Sensitive	Hepatic	Extra-hepatic
SAFF	10.7±.5	5.7±.4	5.0±.3	5.8±.1(54.2)[b]	4.9±.2(45.8)
COCO	7.3±.6	4.2±.3	3.1±.2	4.3±.2(58.9)	3.0±.2(41.1)
Chow	6.3±.4	2.8±.3	3.5±.2	2.7±.1(42.9)	3.6±.2(57.1)

[a]Assayed under standard conditions after pre-incubation for 10' at 27°C in 1.0 mg protamine/ml final assay

[b]Figures in parentheses are the % contribution of each activity to the total activity

the peripheral form of the enzyme. In the experimental group, however, the hepatic form constitutes 54.2% and 58.9% of the SAFF diet and COCO diet-fed animals' post-heparin plasma lipase activity respectively. It should be pointed out, however, that the equations used for the calculation of the two lipase activities has been worked out in the rat model (53). Whether rabbit post-heparin lipase activity behaves similarly has not yet been completely verified. In any event, in normal rat post-heparin plasma a ratio of peripheral to hepatic-lipase activities has been reported to be 1.5:1 (53). In the chow-fed rabbit a ratio of 1.33:1 has been found in the above experiment which seems comparable to the rat figure. The same ratio in rabbits fed either the SAFF or COCO diet was 0.85:1 and 0.70:1 respectively.

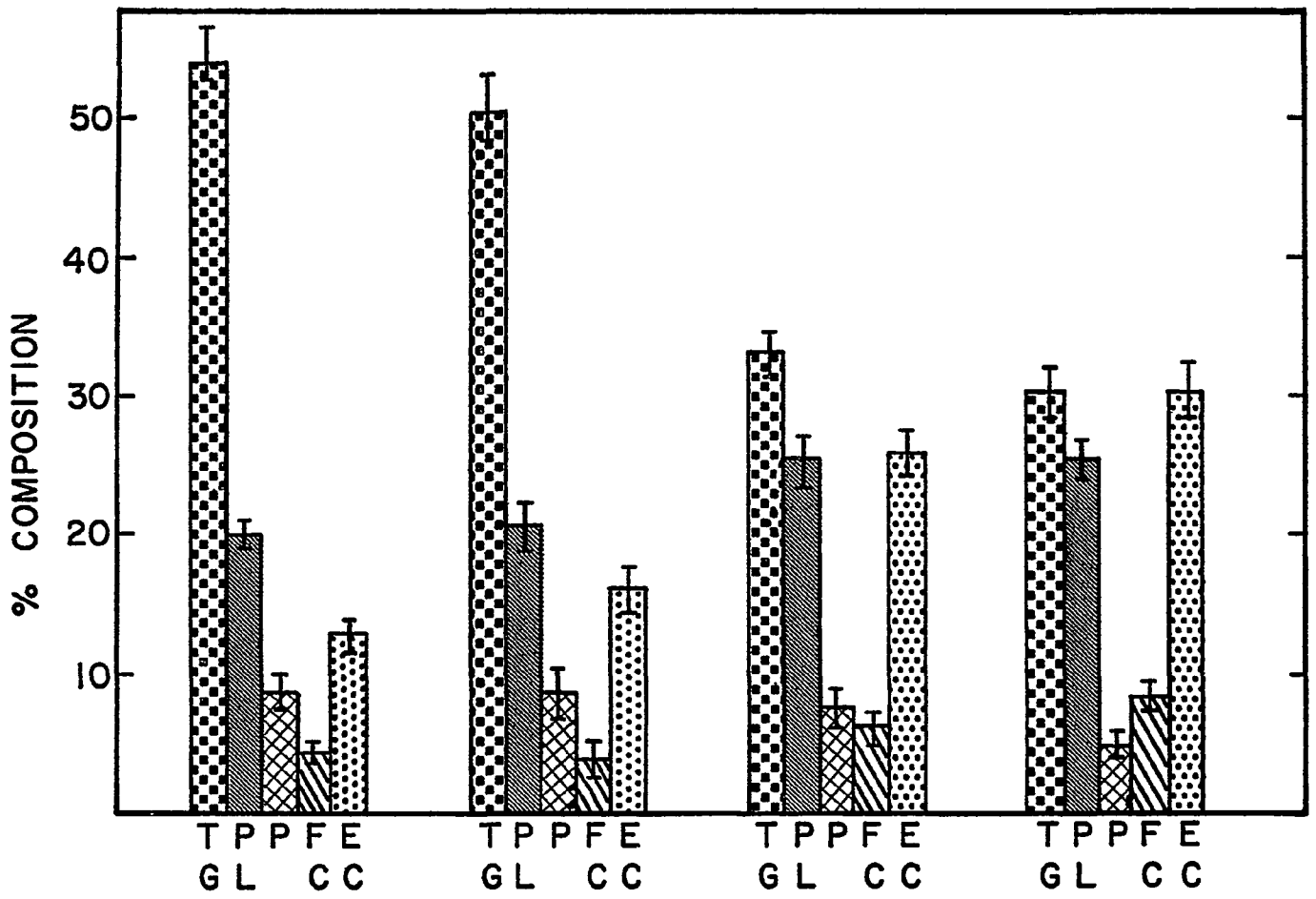
Section III

A. Chemical Composition of VLDL Remnants

The VLDL ($d < 1.006$ g/ml) isolated from rabbits fed either chow or the experimental diets were incubated in vitro with either EHP LPL, PHP LPL, or with normal $d > 1.063$ g/ml plasma from chow-fed animals for the purpose of preparing VLDL remnants (see Materials and Methods). The chemical compositions of the remnants prepared in this fashion are given below.

When chow-fed rabbit VLDL were incubated with normal pre-heparin ($d > 1.063$ g/ml) plasma (NPLAS), no appreciable change in chemical composition of the $d < 1.019$ g/ml fraction, i.e. the "VLDL remnant" isolated after the in vitro incubation, occurred (Figure 21). There was a slight relative shift of core components (cholesteryl ester and triglyceride) possibly due to exchange or transfer with lipoproteins in the $d > 1.063$ g/ml fraction.

Figure 21. Relative chemical composition of normal chow-fed rabbit VLDL, and VLDL after incubation with normal rabbit (d > 1.063 g/ml) plasma (NPLAS), intact rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, PHP LPL) or supradiaphragmatic rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, EHP LPL). See Materials and Methods for conditions of VLDL remnant preparation. Abbreviations used are as described in Figure 1.


% COMPOSITION
50
40
30
20
10
T P P F E
G L C C
T P P F E
G L C C
T P P F E
G L C C
T P P F E
G L C C
ENZYME PREP
NONE
("NORMAL" VLDL)
NPLAS
PHP
EHP

CHOW FED

Yet the surface to core ratio (see Table 15) remained constant (0.50) and the VLDL was otherwise unchanged. Figure 21 also shows that when VLDL remnants were prepared by incubating the chow-fed rabbit VLDL with either the EHP LPL or PHP LPL preparation, 60% of the original VLDL triglyceride was hydrolyzed. Along with this triglyceride depletion, the remnant particle became enriched in cholesteryl ester. Additionally remnant phospholipids were relatively enriched. Yet in absolute terms, loss of phospholipid occurred (from 27 mg/dl lipoprotein to 20 mg/dl lipoprotein). This relative increase in phospholipid is only an apparent one due to the considerable loss of triglyceride from the VLDL particle. Lastly, a loss of protein (from 10 mg/dl lipoprotein to about 5 mg/dl lipoprotein) occurred while the free cholesterol concentration remained the same. No real differences in the chemical composition (Figure 21) or surface to core ratios (Table 15) were observed however between remnants prepared with either EHP LPL or the PHP LPL preparations indicating the possible lack of importance of the hepatic lipase in the formation of remnant lipoproteins.

When either SAFF-fed or COCO-fed rabbit VLDL were incubated with the NPLAS preparation, again no differences in chemical composition were noted (Figures 22 and 23). A slight relative shift of the core components, triglyceride and cholesteryl ester, again occurred but this shift was less pronounced than in the chow-fed rabbit VLDL case. Surface to core ratios remained similar, however, indicating no appreciable change in the VLDL after incubation with the lipase free preparation, NPLAS.

VLDL remnants prepared by incubating SAFF diet or COCO diet-fed rabbit VLDL with either EHP LPL or PHP LPL preparations yielded about 41% and 44% triglyceride hydrolysis respectively, regardless which enzyme preparation

Table 15. Surface to Core Ratios of VLDL Remnants Prepared from VLDL of Various Dietary Treatments and Enzyme Preparations (Ave. 2 Determinations)

Dietary Treatment	Enzyme Preparation			
	None	NPLAS	PHP LPL	EHP LPL
Chow	.49	.50	.72	.70
SAFF	.60	.56	.64	.65
COCO	.60	.57	.53	.51

Figure 22. Relative chemical composition of SAFF diet-fed rabbit VLDL and VLDL after incubation with normal rabbit (d > 1.063 g/ml) plasma (NPLAS), intact rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, PHP LPL) or supradiaphragmatic rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, EHP LPL). See Materials and Methods for conditions of VLDL remnant preparation. Abbreviations used are as described in Figure 1. Standard errors are indicated for n = 5.

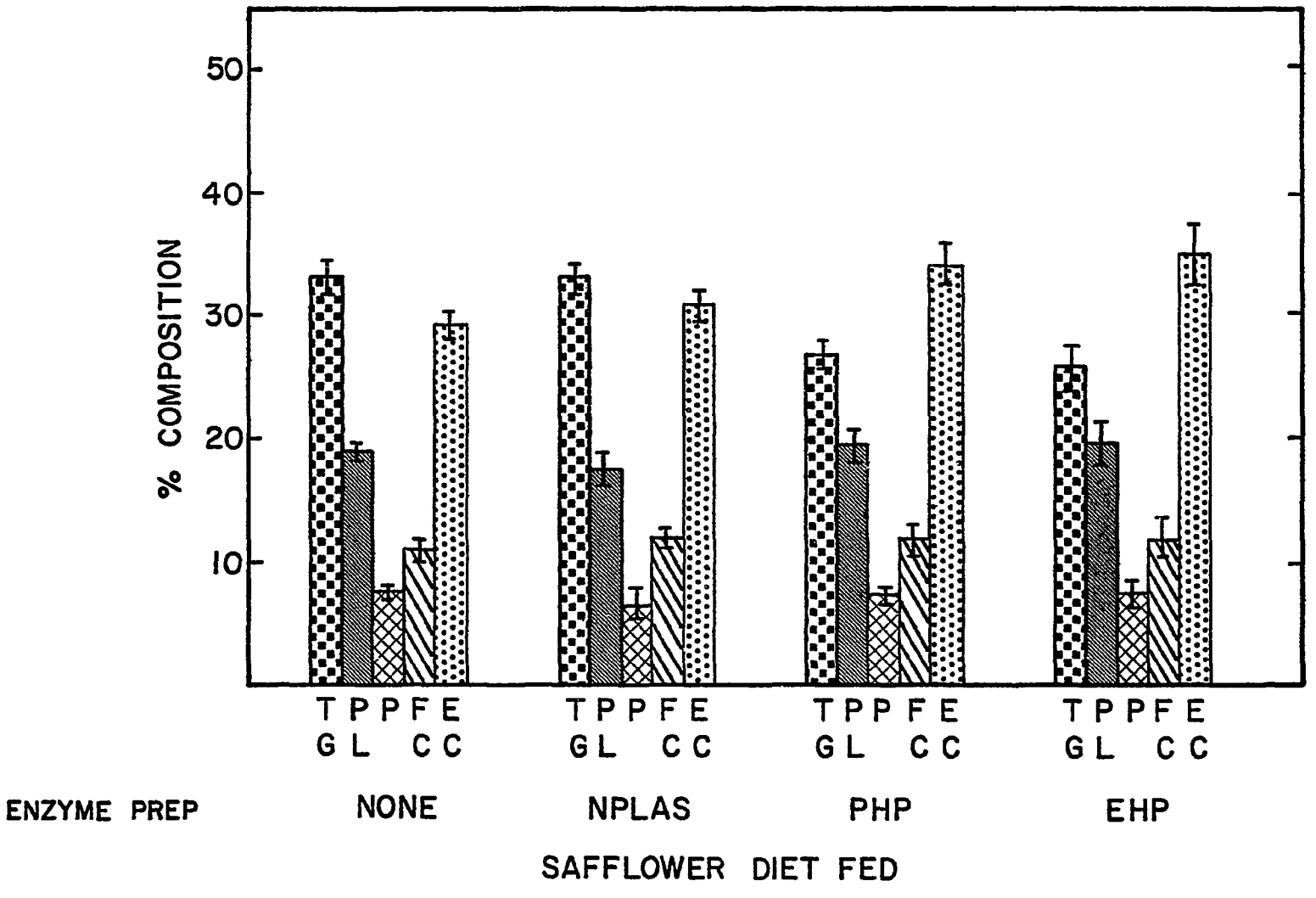
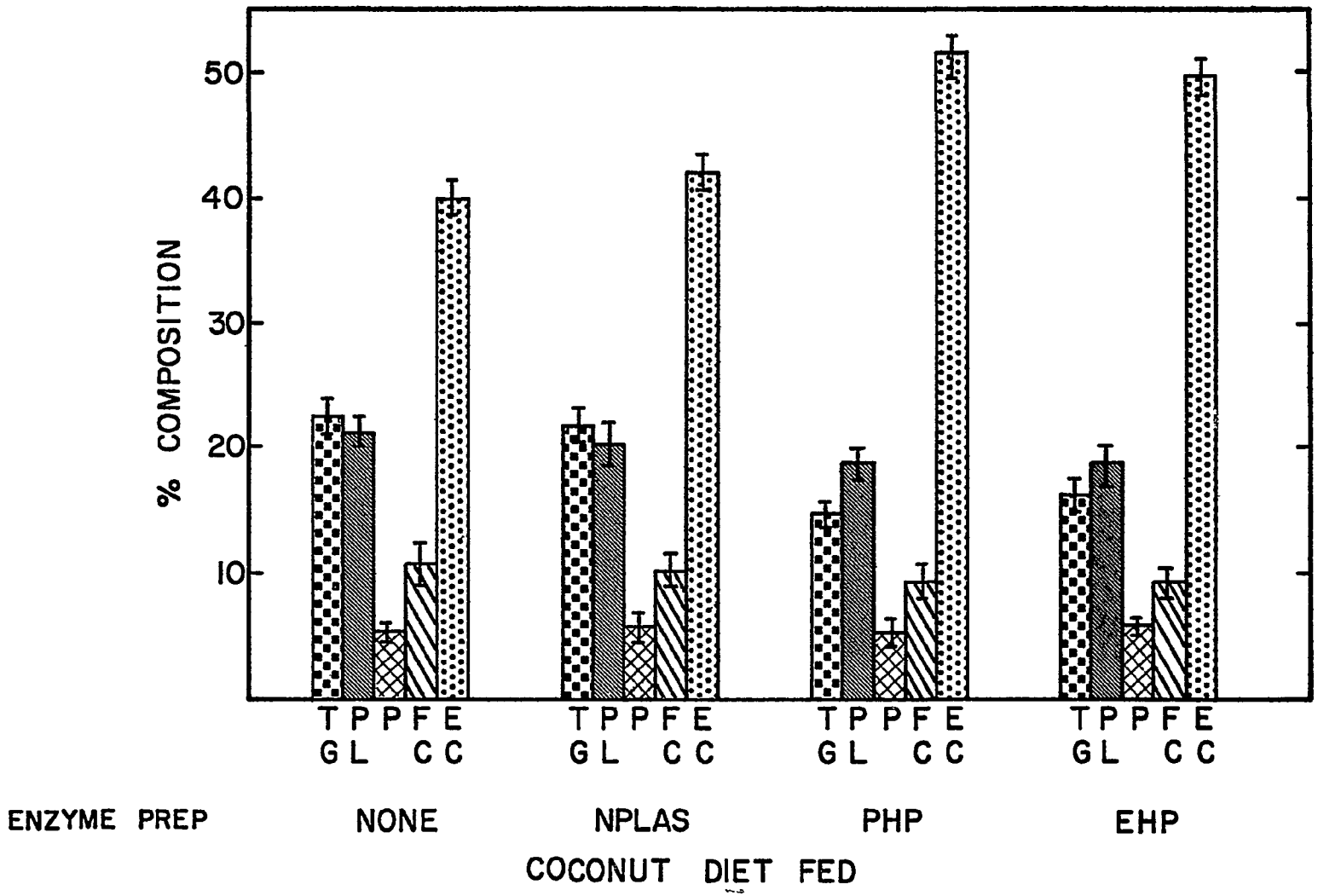

50
40
30
20
10
% COMPOSITION
T P P F E
G L C C
T P P F E
G L C C
T P P F E
G L C C
T P P F E
G L C C
ENZYME PREP
NONE
NPLAS
PHP
EHP
SAFFLOWER DIET FED

Figure 23. Relative chemical composition of COCO diet-fed rabbit VLDL and VLDL after incubation with normal rabbit (d > 1.063 g/ml) plasma (NPLAS), intact rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, PHP LPL) or supradiaphragmatic rabbit post-heparin plasma triglyceride lipase (d > 1.063 g/ml, EHP LPL). See Materials and Methods for conditions of VLDL remnant preparation. Abbreviations used are as described in Figure 1. Standard errors are indicated for n = 5.


50
40
30
20
10
% COMPOSITION
T G
P L
P C
F C
E C
ENZYME PREP
NONE
NPLAS
PHP
EHP
COCONUT DIET FED

was used. These remnant particles became enriched in cholesteryl ester as well. It should be noted that the VLDL from rabbits fed either semisynthetic diet were already appreciably enriched in cholesteryl esters when compared with the chow-fed rabbit VLDL. As in vitro remnant production occurred, the VLDL from rabbits fed the semisynthetic diets became further triglyceride-depleted and cholesteryl ester-enriched. This result indicates the possible existence of a continuum of VLDL remnant particles ranging from those only slightly triglyceride-depleted to ones considerably poor in triglyceride and enriched in cholesteryl ester. Indeed the VLDL from animals fed the experimental diets may actually be a type of remnant of normal triglyceride-rich VLDL which for various metabolic reasons are building up in the bloodstream when experimental diets are fed.

In the case of VLDL remnants prepared from VLDL of rabbits fed the semisynthetic diets, however, both a relative and absolute decrease in phospholipid concentration occurred. Lastly, the absolute level of both protein and free cholesterol may have decreased somewhat in the VLDL remnant when compared to the VLDL itself. The relative amounts of these constituents, however, remained roughly the same (see Figures 22 and 23).

When surface to core ratios of VLDL remnants prepared from VLDL of the various dietary treatments are compared, it can be seen that similar ratios occur whether EHP LPL or PHP LPL enzyme preparations are used for the in vitro incubations (Table 15). As previously indicated, this phenomenon may be due to the fact that the hepatic lipase, present in PHP LPL but not EHP LPL preparations, may offer no significant contribution to remnant formation. When the surface to core ratios are compared between VLDL and its remnant, some differences become apparent. Chow-fed rabbit VLDL was

the largest VLDL observed with a surface to core ratio of 0.50, while the chow-fed rabbit remnant was the smallest particle (surface to core ratio of 0.72). This result is probably due to the depletion of 60% of the VLDL triglyceride and the concomitant relative increase of surface constituents as the triglyceride is lost. The VLDL from rabbits fed the semisynthetic diets is by comparison a smaller particle than is the chow-fed rabbit VLDL (see Table 15, surface to core ratios of 0.60 from either the COCO or SAFF diet rabbit VLDL vs. 0.50 in the chow case).

A comparison of ratios of remnants in the SAFF and COCO diet cases, however, showed the COCO VLDL remnant as having a smaller surface to core ratio (0.52) whereas the SAFF VLDL remnants had a value of 0.64 while both particles lost a similar amount of triglyceride via hydrolysis (40%). A possible explanation is that in the COCO diet-fed rabbit, more cholesteryl ester is present in the core of the remnant VLDL yielding a particle with larger mean diameter, and smaller surface to core ratio.

B. Agarose Gel Electrophoresis of VLDL Remnants: Variation with Diet

Agarose gel electrophoresis of VLDL remnants prepared from VLDL of rabbits fed either the chow or SAFF diets and using the EHP LPL enzyme preparations (Figures 24 and 25) predominately showed β-mobility. Remnants prepared using the PHP LPL enzyme preparation, however, showed a mobility more intermediate between the typical beta and pre-beta, indicating that apolipoproteins are either being lost or perhaps altered making the particle somewhat more positively charged. VLDL incubated in the presence of normal $d > 1.063$ g/ml plasma (NPLAS) maintained its pre-β mobility although some exchange or transfer of apolipoproteins (apo C) may have occurred.

Figure 24. Densitometric scans of VLDL and VLDL remnants after agarose electrophoresis from rabbits fed the chow diet indicating their relative mobilities. Conditions were as described in Figure 6. Abbreviations are as follows: VLDL, very low density lipoprotein; EHP, VLDL remnant formed after incubation of VLDL with the supradiaphragmatic rabbit enzyme preparation; PHP, VLDL remnant formed after incubation of VLDL with the intact rabbit enzyme incubation of VLDL with the intact rabbit enzyme preparation; NPLAS, VLDL "remnant" formed after incubation of VLDL with the normal rabbit plasma preparation.

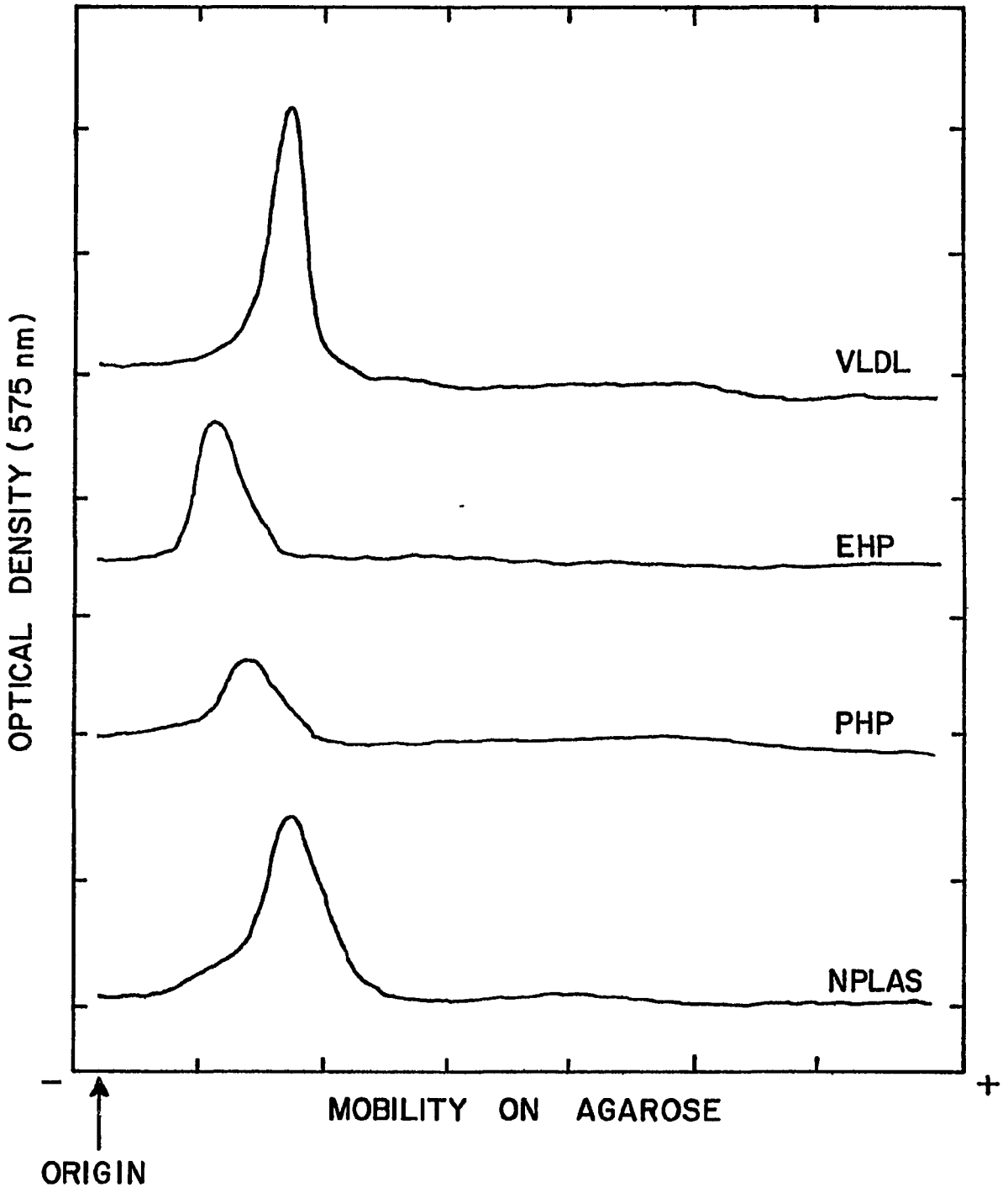
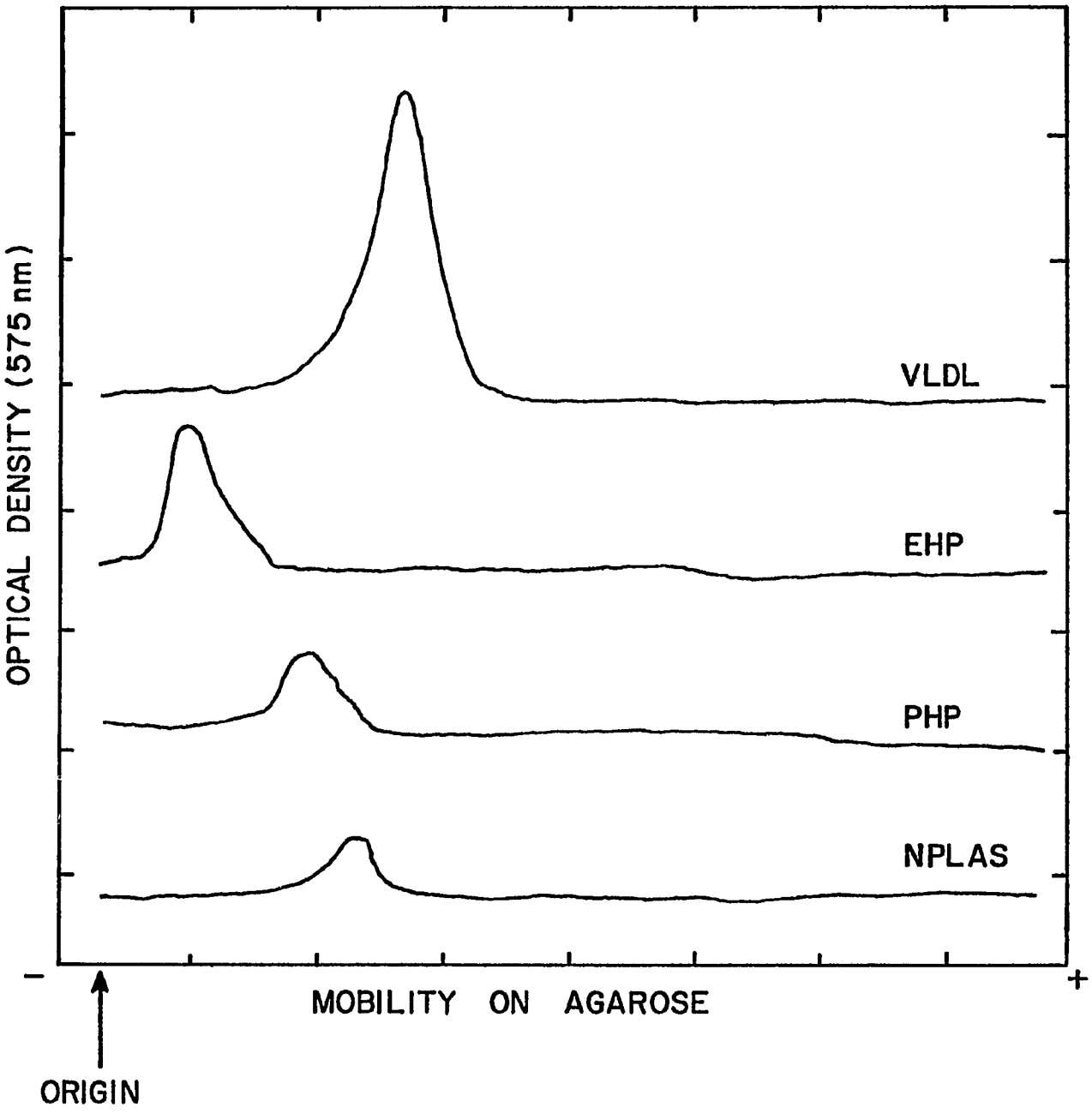

OPTICAL DENSITY (575 nm)
VLDL
EHP
PHP
NPLAS
−
+
MOBILITY ON AGAROSE
ORIGIN

Figure 25. Densitometric scans of VLDL and VLDL remnants after agarose electrophoresis from rabbits fed the SAFF diet indicating their relative mobilities. Conditions and abbreviations are as described in Figure 24.


OPTICAL DENSITY (575 nm)
VLDL
EHP
PHP
NPLAS
-
+
MOBILITY ON AGAROSE
ORIGIN

Likewise when electrophoresis was performed on VLDL remnants prepared from VLDL of rabbits fed the COCO diet, β-mobility occurred (Figure 26). It should be noted, though, that the VLDL from these animals showed β-mobility initially so no real net change in mobility occurred.

The change in mobility from pre-beta to beta might be explained due to the loss of apolipoprotein C from the VLDL as triglyceride hydrolysis proceeded. In addition, as shall be discussed in the next section, a relative, and in some cases an absolute, increase in the arginine-rich lipoprotein (apo E) also occurred. The net charge on the protein portion of the VLDL remnant was probably rendered more positive and less mobile in the agarose gel. It can be concluded, however, that as VLDL remnants are produced, a lipoprotein particle more closely resembling LDL in its electrophoretic behavior on agarose gel occurs.

C. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of VLDL Remnants: Variation with Diet

After VLDL remnants were prepared <u>in vitro</u> from VLDL of rabbits fed the various diets, an aliquot of each was taken for the purpose of SDS-polyacrylamide gel electrophoresis. Densitometric scans at 675 nm were made of the gels after Coomassie blue staining and the areas under the curves corresponding to the apolipoprotein content was determined. Table 16 contains the results of these experiments. When VLDL from rabbits fed any of the three diets was incubated with the NPLAS preparation, no marked changes in apolipoprotein composition were noted when compared with the apolipoprotein composition of the respective VLDL used. There was some loss of apo C peptides, however, probably due to transfer or exchange reactions with the $d > 1.063$ lipoproteins present in the enzyme preparation.

Figure 26. Densitometric scans of VLDL and VLDL remnants after agarose electrophoresis from rabbits fed the COCO diet indicating their relative mobilities. Conditions and abbreviations are as described in Figure 24.

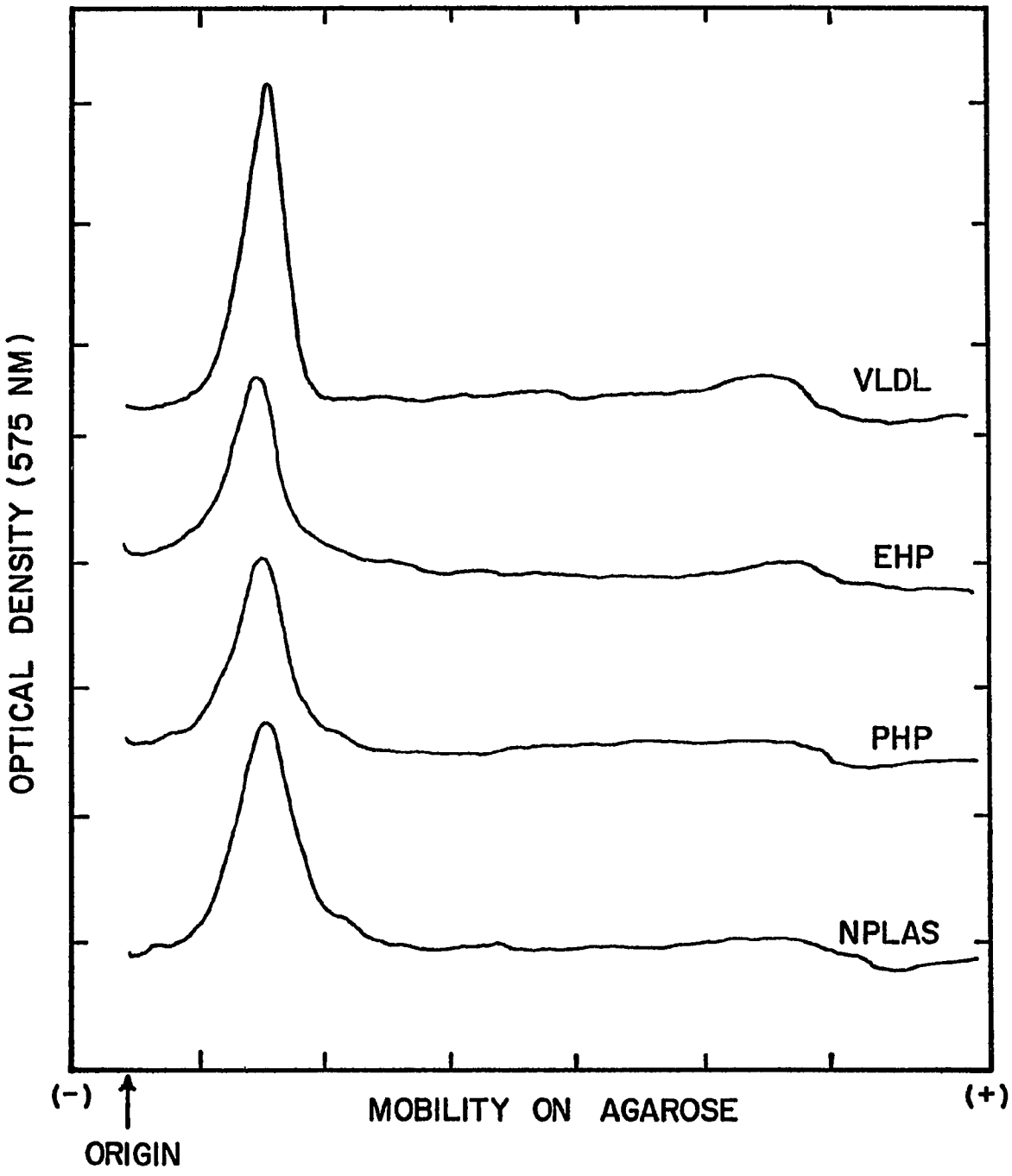

OPTICAL DENSITY (575 NM)
VLDL
EHP
PHP
NPLAS
(-)
ORIGIN
MOBILITY ON AGAROSE
(+)

Table 16. % Composition of Apo VLDL Remnants Prepared from VLDL of Rabbits Fed Various Diets (Ave. 2 Determinations)

Diet	Remnant Preparation	Apo B	Arg-Rich	Apo C
Chow	None (VLDL)	60	8	32
	NPLAS	62	8	30
	EHP	76	9	15
	PHP	75	9	16
SAFF	None (VLDL)	58	17	25
	NPLAS	61	15	24
	EHP	71	18	11
	PHP	70	16	14
COCO	None (VLDL)	45	39	16
	NPLAS	51	36	14
	EHP	60	35	5
	PHP	62	34	4

When the EHP LPL or PHP LPL enzyme preparations were utilized for the preparation of remnant lipoproteins, a marked loss of apo C peptides occurred with a relative increase in the apo B peptides. In the case of remnants prepared from VLDL of rabbits fed either the chow or the SAFF diet, relative increases in the arginine-rich peptide of the apolipoprotein also occurred. The significance of these results is that as remnants are formed, the apo B and arginine-rich peptides are retained, while apo C peptides are preferentially lost, possibly due to exchange or net transfer to HDL in the incubation media.

When VLDL remnants prepared from rabbits fed the COCO diet were subjected to SDS-polyacrylamide gel electrophoresis, however, a relative decrease in apo C and a slight decrease in the arginine-rich peptide occurred. It appears, therefore, that while the apo B peptide is retained in the remnant particle, some loss of the arginine-rich peptide is taking place. It remains to be determined whether this slight loss is statistically significant, since the electrophoresis performed is the result of only two determinations.

D. Fatty Acid Composition of Lipid CLasses of VLDL Remnants: Variation with Diet

The results of fatty acid analysis of VLDL remnant lipids after incubation with the PHP LPL or EHP LPL preparations or with the normal plasma preparation are given in Tables 17, 18 and 19.

From the fatty acid compositional data obtained, it appeared that no real differences exist as far as the phospholipid and cholesteryl ester fractions are concerned. Cholesteryl esters have been reported to transfer between HDL and VLDL in human plasma (110), so whatever differences might

Table 17. Fatty Acid Analysis (Mean %) of Lipid Classes of VLDL Remnants Prepared from VLDL of Rabbits Fed the Chow Diet: Variation with Enzyme Preparation (Average of Two Determinations)

	Triglycerides			Phospholipids		Cholesteryl Esters	
Fatty Acid	NPLAS	PHP	EHP	NPLAS	EHP	NPLAS	PHP
12:0	-	-	-	-	-	-	-
14:0	3.9	3.2	4.1	1.4	1.7	1.4	2.6
14:1	-	-	-	-	-	-	-
16:0	33.5	41.4	42.2	34.8	36.2	19.1	22.4
16:1	5.4	4.0	tr	3.1	1.0	4.7	2.9
18:0	6.1	10.3	9.1	21.3	21.2	3.7	6.3
18:1	35.9	33.5	37.2	15.2	36.4	44.0	39.0
18:2	13.6	6.8	6.0	18.2	4.0	25.2	23.0
>18:2 <20:4	3.9	tr	1.0	0.5	tr	tr	3.0
20:4	tr	0.5	tr	-	0.5	tr	0.6

tr refers to trace amount (<0.5%)

Table 18. Fatty Acid Analysis (Mean %) of Lipid Classes of VLDL Remnants Prepared from VLDL of Rabbits Fed the SAFF Diet: Variation with Enzyme Preparation (Average of Two Determinations)

	Triglycerides			Phospholipids			Cholesteryl Esters	
Fatty Acid	NPLAS	PHP	EHP	NPLAS	PHP	EHP	NPLAS	EHP
12:0	-	-	-	-	-	-	-	-
14:0	1.4	2.7	1.7	-	-	-	-	-
14:1	-	-	-	-	-	-	-	-
16:0	34.8	44.2	36.2	31.8	33.2	11.7	20.4	19.0
16:1	3.1	1.9	1.0	tr	tr	3.6	3.4	9.7
18:0	21.3	25.9	21.2	27.1	26.2	31.8	4.6	4.0
18:1	15.2	10.9	36.4	14.2	9.2	23.7	42.9	31.7
18:2	18.2	11.9	4.0	26.9	32.1	28.6	28.7	35.6
>18:2 <20:4	0.5	1.0	tr	-	tr	-	-	-
20:4	-	2.0	0.5	tr	tr	tr	tr	-

tr refers to trace amount (<0.5%)

Table 19. Fatty Acid Analysis (Mean %) of Lipid Classes of VLDL Remnants Prepared from VLDL of Rabbits Fed the COCO Diet: Variation with Enzyme Preparation (Average of Two Determinations)

	Triglycerides			Phospholipids			Cholesteryl Esters		
Fatty Acid	NPLAS	PHP	EHP	NPLAS	PHP	EHP	NPLAS	PHP	EHP
12:0	-	-	-	-	-	-	1.2	-	tr
14:0	17.7	18.4	10.2	1.1	9.6	1.7	5.0	4.1	4.9
14:1	-	-	1.3	-	1.8	-	-	-	-
16:0	38.5	37.5	36.4	33.0	30.4	30.3	24.8	32.0	28.1
16:1	6.5	6.3	3.9	4.6	6.2	1.8	6.2	2.3	6.9
18:0	11.8	11.8	20.0	26.0	23.6	28.2	10.3	7.8	10.9
18:1	24.4	23.8	28.7	17.9	18.2	19.6	42.0	43.4	40.0
18:2	1.2	2.2	3.6	17.3	7.8	18.4	10.6	9.7	9.1
>18:2 <20:4	-	-	-	-	2.3	-	tr	0.7	-
20:4	tr	tr	tr	tr	tr	-	tr	tr	-

tr refers to trace amount (<.05%)

exist in the fatty acid composition may be at least partially the result of transfer or exchange reactions since HDL was present in the enzyme preparations used. This phenomenon is also true with regard to the phospholipid fractions as well, but in addition, phospholipase activity associated with the lipoprotein lipase enzyme could also be responsible for fatty acid composition differences.

While this data represents only the average of two determinations, it appears that, in the phospholipid and cholesteryl ester classes, minor variations among the content of oleic acid in the SAFF and chow diet cases and among the linoleic acid in the COCO diet case occurred. VLDL remnants prepared by incubating VLDL with the $d > 1.063$ g/ml fractions of normal plasma also showed some small fatty acid composition differences as well.

In the case of the relative fatty acid content of the triglyceride fraction of VLDL remnants, a few trends became apparent. In the SAFF (and chow) cases, it appeared that a loss of linoleic acid (18:2) occurred during remnant preparation, while the saturated fatty acids, palmitate and stearate increased only slightly in relative content. This trend was not observed, however, in the COCO diet case.

IV. DISCUSSION

The semisynthetic diet, high in saturated fat (COCO), produced a marked effect on the serum lipid concentrations of rabbits. When compared with the high unsaturated fat semisynthetic diet (SAFF), the COCO diet produced a notable hypercholesterolemia with both plasma free and esterified cholesterol being elevated. Plasma lipoproteins showed an increase of serum VLDL, IDL and LDL in the COCO diet case as well (i.e. those lipoproteins with $d < 1.063$ g/ml). Only a slight decrease in serum HDL levels was observed

after feeding the experimental diets but the HDL level was lowest in the COCO diet case. The COCO diet used has been shown to be atherogenic in rabbits by others (80-83) and indeed when thoracic aorta segments from representative animals of each dietary group were stained with fat red 7B, the ones from rabbits fed the COCO diet had more grossly visible atherosclerotic plaque accumulations than aortas from SAFF-fed animals, which contained some lesions, or from chow-fed rabbits, which showed no gross lesions.

Since the animals in this study were used as their own controls, only two rabbits were sacrificed after feeding the SAFF diet and before the COCO diet was begun. Consequently assessing the degree of atherogenicity of the two experimental diets grossly was difficult. Yet in comparing the few aortas which were dissected and stained, it was felt that the COCO diet yielded larger, more extensive areas of plaque accumulation than after feeding the SAFF diet. The results of the blood chemistries (i.e. hypercholesterolemia) and the differences in the characteristics of the serum lipoproteins of rabbits fed the two diets further strengthens the concept of the high saturated fat diet as being more atherogenic. It should be mentioned that the SAFF diet, while producing fewer changes in the serum cholesterol and lipoprotein concentrations, is also atherogenic when compared to chow. A possible explanation of this is not only due to the high fat loading of the rabbits via the diet, but also due to the presence of casein and sucrose, which are more atherogenic materials than other protein and carbohydrate sources (86, 111). The important difference is that when hydrogenated coconut oil is used as the fat source, more significant changes are occurring. These changes will next be discussed.

Numerous investigators have found that the serum VLDL of hypercholesterolemic rabbits are rich in cholesteryl ester and relatively poor in triglyceride (21, 78). This effect has been shown in rabbits supplemented with either cholesterol alone or cholesterol plus fat from various sources. Evidence is presented here for the occurrence of cholesteryl ester enriched VLDL ($d < 1.006$ g/ml) in rabbits fed semisynthetic diets totally free of cholesterol and high in fat. The cholesterolemic effect of these diets is more marked in the case of feeding highly saturated fat than when the polyunsaturated fat diet was fed.

The five major changes consistently observed in the serum lipoproteins after cholesterol feeding (60) have been compared to the result of feeding both the COCO and SAFF diets to rabbits (see Results, Table 9). In the COCO diet case, HDL_C was not found to be present among the serum lipoproteins. One possible explanation for the occurrence of HDL_C, when it does occur in the cholesterol-induced hyperlipoproteinemia situation, is that the HDL_C are formed by an overloading of typical HDL with cholesterol (60). When the COCO diet was fed, serum cholesterol values, while significantly high when compared with chow, were not as high as when cholesterol supplementation is used. Thus cholesterol overloading has probably not yet occurred. This difference, therefore, may help to explain the initial changes in lipoprotein alteration and serum chemistries seen in the atherogenic process. The HDL_C may occur at a later stage of the disease.

In order to begin to pinpoint which factor or factors might be involved in the increased atherogenicity of the COCO diet over the SAFF diet, a comparison of the entries in Table 9 of Results is helpful. The lipoproteins, IDL, HDL and HDL_C all show no marked difference whether the COCO or SAFF

diet was fed (IDL increases, HDL decreases, and HDL_c is absent). However, in the COCO-fed case, B-VLDL is present and LDL is increased, while when the SAFF diet is fed, no B-VLDL is seen and LDL actually decreases somewhat. Although increases in LDL levels have been implicated to a large extent in atherogenesis (45, 77), the presence of altered VLDL, the B-VLDL, may indeed be equally as important, especially since a precursor-product relationship between VLDL and LDL has been demonstrated (60, 73, 112).

In summary, the beta-mobility of the COCO diet-fed rabbit VLDL seen on agarose gels in this study indicates the presence of an altered lipoprotein particle which may be important in atherogenesis. While the SAFF diet-fed rabbit VLDL's chemical composition was altered toward a cholesteryl ester rich particle, its mobility on agarose remains pre-beta. In addition, the serum cholesterol and cholesteryl ester concentration in the case of rabbits fed the SAFF diet, while higher than the chow-fed animals' sera, was appreciably lower than serum after the COCO diet feeding. Lastly, LDL levels in rabbits fed the SAFF diet were about the same as in the chow-fed case, while an increased LDL concentration was seen when the COCO diet was fed. Thus, the SAFF diet appears to be exerting some degree of prevention against hypercholesterolemia, hyperlipoproteinemia, and VLDL modification, all of which when present may be atherogenic.

This effect may be explained by the finding that when saturated fatty acids are used to perfuse isolated rat liver (68), the molar ratio of cholesterol to triglyceride in the VLDL secreted was greater than when either oleic or linoleic acids were used. Thus the COCO diet, by virtue of its high saturated fat content, resulted in the stimulation of cholesterogenesis and the subsequent incorporation of this cholesterol into the secreted lipoprotein.

Consistent with this presumed increased amount of cholesterol synthesis is the finding that both different relative and absolute amounts of apolipoproteins are present in the VLDL of rabbits fed the experimental diets. One particular apo VLDL peptide was markedly increased with respect to the other apo VLDL peptides normally present in VLDL. This peptide is similar in mobility on polyacrylamide gels to that found by Shore et al. (78) in cholesterol-fed rabbits which was determined to be arginine-enriched. In the COCO diet case, this peptide accounted for almost 40% of the total VLDL apoprotein content, nearly a five-fold increase when compared to the chow diet. A similar result was observed, but to a lesser extent, after the feeding of the SAFF diet to the rabbits.

This finding may be responsible for the decreased mobility of the COCO diet VLDL on the agarose gels in that the greater amount of the arginine-rich peptide, a positively charged species at the pH employed, migrates less toward the anode during electrophoresis than normal VLDL. Additionally, this observation is consistent with the suggestion made by Shore et al. (78) that the arginine-rich peptide is one of the apolipoproteins primarily involved with cholesterol transport and metabolism. The parallel increases in cholesteryl esters in the VLDL and the arginine-rich peptide(s) is suggestive of the peptide functioning as a binding protein specific for cholesteryl esters (or some complex of these esters with other lipids).

In any event, the diet-induced situation in the rabbit reported here appears similar to findings reported after the cholesterol feeding of rabbits (78) and it may be analogous to the lipoprotein profile seen in human type III hyperlipoproteinemia (73). In this condition early atherosclerotic

lesions are accompanied by an increased amount of cholesteryl esters in the VLDL and contain increased amounts of the arginine-rich peptide as well. The significance of these present studies, therefore, is that while no dietary cholesterol was provided, in response to a high saturated fat diet rabbits assembled altered VLDL with similar properties as VLDL made under conditions of both cholesterol-induced hyerplipoproteinemia and human type III hyperlipoproteinemia.

Zilversmit has concluded that the lipoproteins which are high in cholesteryl ester and poor in triglyceride appear to originate from either chylomicrons or VLDL and are possibly important in atherogenesis (118, 119). These particles may indeed be atherogenic, yet the results of experiments presented here and those of Shore et al. (78) as well suggest that the remnants prepared from the d < 1.006 g/ml serum lipoprotein fraction may be derived from VLDL which has originated via liver synthesis.

According to Zilversmit (21), two-thirds of the cholesteryl ester component of VLDL is derived from chylomicron degradation. While this cholesteryl ester is of intestinal origin, these results do not necessarily support the idea that these particles are the direct result of chylomicron metabolism. Cholesteryl esters have been reported to exchange and transfer between HDL and VLDL (110). In addition, chylomicron remnant cholesteryl ester is metabolized in the liver (113) and these components may then be reassembled into VLDL and perhaps HDL as well. Consequently, the altered VLDL may not be chylomicron remnants per se even though part of their cholesteryl ester component has its origin in the chylomicron.

Lastly, the apolipoprotein composition of the altered VLDL also indicates that these particles are not merely remnants of chylomicron or VLDL

catabolism. In the present studies, VLDL remnants prepared in vitro from chow-fed rabbit VLDL, while lower in relative apo C content, showed no appreciable increase in the arginine-rich peptide. Other studies (114-118) have shown that after cholesterol supplementation of experimental animals, altered VLDL are observed with a high arginine-rich peptide content as well. Thus, the altered VLDL may be derived from a unique VLDL species enriched in the arginine-rich peptide and originating in the liver.

After the intravenous injection of heparin, it is possible to measure in plasma the activities of two different triglyceride hydrolases (triglyceride lipase): lipoprotein lipase from peripheral tissues and hepatic lipase.

Enzyme preparations from both intact chow-fed rabbits (containing peripheral and hepatic lipases) and supradiaphragmatic chow-fed rabbits (containing the peripheral lipase) were assayed with VLDL from the various diets as substrate. When VLDL from chow-fed animals was used as substrate, lipase activities were appreciably higher in each case. This finding indicates that the VLDL from experimentally-fed animals is not as effective a substrate for the triacylglycerol hydrolase enzyme as the chow-fed rabbit VLDL. Consequently a build-up of partially catabolized VLDL may be occurring in the circulation of animals when fed the experimental diets. The observation that serum from the COCO diet-fed animals was lipemic is consistent with this concept. A second explanation is that the VLDL of rabbits fed the experimental diets is cholesteryl ester-enriched and triglyceride-poor (both of these molecules are core constituents of VLDL). Consequently, the triglyceride component may somehow not be as available to the enzyme as normal chow VLDL triglyceride.

When surface to core ratios are calculated from compositional data of lipoproteins, a rough idea of particle size can be obtained (35, 72). In general, the larger the ratio, the smaller the respective particle will be.

Lipase activities have been reported to be greater when VLDL or chylomicron particle size is larger (120) (i.e. when they contain more triglyceride in relation to their surface constituents). The activities of the enzyme preparations used in this study should therefore be equal as far as the VLDL substrate from either the SAFF or COCO-fed animals is concerned and indeed they are. Further, when chow-fed rabbit VLDL is the substrate, which has a lower surface to core ratio than either COCO or SAFF diet-fed VLDL, higher lipase activities were observed, attesting to the usefulness of the surface to core ratio in predicting lipase activity.

The two enzyme preparations from different sources were characterized, and were found to exhibit similar properties under the assay conditions employed. Linearity of the activity, pH optima, and rate limiting substrate concentrations were all nearly identical whether the intact or supradiaphragmatic rabbit preparation was assayed. The peripheral lipase, however, is inhibited by 1.0 M NaCl and by protamine (55) while the hepatic lipase is insensitive to these conditions (121). Consequently, the supradiaphragmatic enzyme preparation showed nearly 100% inhibition of lipase activity when preincubated with either 1.0 M NaCl or 1.0 mg protamine sulfate. The enzyme preparation from intact rabbits, as expected, was only partially inhibited due to contributions of enzyme activities from the peripheral tissues as well as the liver.

When the selective measurement of hepatic and peripheral enzyme activities of intact rabbits fed the experimental diets was made and compared

with chow-fed animals, a change in the relative contributions of the two lipase activities to the total activity became apparent. In chow-fed animals, more than half of the total enzyme activity was due to the peripheral lipase. In the experimental groups, it was the hepatic lipase which accounted for more than half of the total post-heparin plasma lipase activity. What may have happened in this case is either an absolute increase in the hepatic lipase or a decrease in the activity of the peripheral enzyme. In the rat, is has been shown that the ratio of peripheral to hepatic lipase in post-heparin plasma is 1.5:1 (53) whereas in these experiments a value of 1.33:1 was calculated. This same ratio of activities in rabbits fed either the SAFF or COCO diet was 0.85:1 and 0.70:1 respectively. Whether these ratios correspond to a decrease in peripheral lipase activity or an increase in the hepatic form awaits the methodology of determining absolute triglyceride lipase activities in post-heparin plasma. It does appear, however, that since an increase in the d < 1.063 g/ml lipoprotein fraction has occurred in the experimental diet-fed case, a decrease in the peripheral lipase, important in the catabolism of these particles, may have occurred.

Although the hepatic lipase has the capability of hydrolyzing a variety of substrates (e.g. monoglyceride, diglyceride, triglyceride and phospholipid) in vitro, it is presently an enzyme in search of function with regard to lipoprotein metabolism in vivo (112). Consequently, it was felt that the in vitro preparation of VLDL remnants using both intact and supradiaphragmatic enzyme preparations might shed some further light in the role of the hepatic lipase. However, no real differences in the chemical composition or surface to core ratios were observed between remnants prepared with either the supradiaphragmatic or intact rabbit enzyme preparations. This result is

consistent with the suggestion that the hepatic lipase is not important in the formation of remnant lipoproteins (122).

The preparation of VLDL remnants from VLDL of rabbits fed the various diets was carried out in order to provide further information about the catabolism of both the altered and normal lipoprotein particles obtained via the dietary manipulation. The VLDL remnants prepared from chow-fed rabbit VLDL were triglyceride-poor and cholesteryl ester-enriched. Loss of phospholipid and protein occurred while the free cholesterol concentration remained unchanged. In addition, the mobility of the remnant lipoproteins on agarose was, for the most part, beta. By contrast, the VLDL from which these remnants were prepared were pre-beta in mobility indicating some loss or alteration of the protein moiety. Lastly, the surface to core ratios indicate that the remnant lipoprotein is a smaller particle than the VLDL from which it was prepared.

These results suggest that the cholesteryl ester-rich, triglyceride-poor VLDL observed after feeding the COCO or SAFF diets may be remnants of triglyceride-rich particles which are building up in the circulation (73). However, as previously discussed, the relative composition of the apolipoproteins of VLDL from the experimental diet-fed animals and that of the VLDL remnants from chow-fed rabbits is incompatible with this concept. While apo C peptides are lost from the VLDL as remnants are formed, the content of arginine-rich peptide(s) in the chow-fed rabbit VLDL remnants is in no way as high as that in the VLDL from the COCO diet (or for that matter the SAFF diet). Therefore, since the arginine-rich protein is only present in small amounts (<10% of the total apolipoprotein) in serum VLDL, it is likely to be present in only small amounts in remnant particles

formed from this VLDL. Additionally, surface to core ratios are not a completely accurate measure of particle size (35) and the slight differences found among the normal and altered VLDL may not be real. A more accurate measure of particle size is via electron microscopy. The work of Shore et al. (78) has shown that VLDL from rabbits made hyperlipoproteinemic with cholesterol has a larger mean particle diameter than normal chow-fed rabbit VLDL. If the experimental diet-fed rabbit VLDL consisted primarily of VLDL remnants, they would be expected to be smaller, not the same size or larger, than the VLDL from which they are formed. Further work is needed (i.e. electron microscopy) before a definite conclusion in the case of the experiments reported here can be reached.

The VLDL from either SAFF-fed or COCO-fed rabbits is already appreciably cholesteryl ester-enriched, and triglyceride-poor. Yet when remnants were prepared using either of these VLDL, it became further triglyceride-depleted and cholesteryl ester-enriched. This result suggests the existence of a continuum of VLDL and remnant particles ranging from those only slightly triglyceride-depleted to ones considerably poor in triglyceride and rich in cholesteryl esters.

Both the COCO and SAFF diet-fed rabbit VLDL had similar surface to core ratios. Compositional analyses of the lipoprotein showed the VLDL from the SAFF diet case to contain more triglyceride and less cholesteryl ester than the VLDL obtained after feeding the COCO diet. Yet the sum of these two core components was identical in each case. Remnants prepared from these VLDL yielded particles with different surface to core ratios, the COCO diet VLDL being smaller. Since both VLDL lost 40% of their respective triglyceride content, the additional amount of cholesteryl ester in the case

of the COCO diet VLDL remnants may account for the larger mean diameter of this particle. The size of these particles may be related to their accumulation in the circulation in the event that their metabolism and subsequent utilization is somehow slowed or prevented.

The fatty acid composition of the VLDL, as expected, was found to reflect that of the diets fed. In general, the percentages of palmitate and oleate were significantly decreased during the feeding of the polyunsaturated fat diet (SAFF) while linoleate was consistently increased in each lipid class.

It has been previously reported that when rat liver was perfused with palmitic acid, the secreted VLDL had a lower percentage of triglyceride and a higher percentage of cholesterol and phospholipid than did the lipoprotein produced when oleate or linoleate was infused (123). It remained to be determined whether these observations could be extended to a general relationship describing the effects of saturated and unsaturated free fatty acids on the production and properties of VLDL. The fatty acid and chemical compositional data of the VLDL obtained after feeding the SAFF and COCO diets in these experiments are in agreement with these <u>in vitro</u> liver perfusion experiments. When the saturated fat diet was fed, less triglyceride and more phospholipid and cholesterol were present in the resultant VLDL. Additionally, this VLDL triglyceride contained proportionately more palmitic acid than the SAFF diet-fed rabbit VLDL which was enriched in linoleic acid.

It is probable that phospholipid and cholesterol are required to effect some specific configuration of the VLDL which, along with the apopeptides, allows it to carry the fat soluble triglyceride in the aqueous environment of the blood. The polar, hydrophilic groups of these molecules undoubtedly

participate in this function. Palmitic acid (mp 63.1) is solid at room temperature and has a higher melting point than does oleic acid (mp 13.4) or linoleic acid (mp 16.5). Furthermore, the solubility of linoleic or oleic acid in polar solvents exceeds that of palmitic acid. These differences in physical properties may mean that more of the polar cholesterol and phospholipid are required to stabilize the VLDL when it contains a greater proportion of its triglyceride fatty acids as palmitate than linoleate.

In summary, then, since diets high in saturated fats preferentially yield VLDL which contains a higher ratio of cholesterol and phospholipid to triglyceride, and since these lipoproteins may be either used at a slower rate (as previously mentioned) or in some way allowed to build up, it might help explain both the lipid lowering effect of unsaturated fat diets and the more atherogenic nature of the COCO diet used in this study.

Only minor differences in the fatty acid composition of the VLDL remnants occurred when compared to its respective VLDL. One observation, however, warrants further consideration. In the SAFF (and chow) fed cases, a loss of linoleic acid occurred during remnant preparation while the saturated fatty acids, palmitate and stearate increased slightly in relative content. This result was not observed in the COCO diet case indicating no chain-length specificity of the lipase under these conditions. Chain-length specificity of the triglyceride lipase enzyme has not been thought to be a characteristic of this system. Recent work by Hulsmann et al. (124), however, has indicated a higher rate of hydrolysis of monolinoeoylglycerol by a heparin releasable liver lipase than when monopalmitoylglycerol was used. Under the conditions of high polyunsaturated fat diets used in the present study, there may be

some preferential hydrolysis of linoleic acid by the triglyceride lipase preparation as well. This preferential hydrolysis may contribute to the faster utilization of polyunsaturated fatty acids in vivo and consequently to the hypolipidemic effect of dietary polyunsaturated fats.

V. SUMMARY

New Zealand white rabbits were successively maintained on normal chow, a semisynthetic diet high in polyunsaturated fat (safflower oil; SAFF diet) and a semisynthetic diet high in saturated fat (hydrogenated coconut oil; COCO diet) for 9 weeks each.

The β-mobility of VLDL on agarose from rabbits fed the semisynthetic diet high in saturated fat and containing no cholesterol indicates the presence of an altered lipoprotein particle which may be atherogenic. In addition, this VLDL was cholesteryl ester-enriched and triglyceride-poor. This alteration is perhaps regulated by the stimulation of de novo cholesterogenesis in the liver. Although a similar effect has been reported after the cholesterol feeding of rabbits, the significance of the present studies is that while no dietary cholesterol was provided, an altered VLDL is produced with similar properties as those seen under conditions of both cholesterol-induced and human type III hyperlipoproteinemias.

Other effects of this diet are an increase in the $d < 1.063$ g/ml fraction of lipoprotein especially VLDL and LDL_2 and a decrease in the HDL fraction. Hypercholesterolemia is also observed.

When a similar diet, high in polyunsaturated fat, was fed, the VLDL also became cholesteryl ester-enriched and triglyceride-poor but it remained pre-beta in its mobility on agarose gels. In addition, this diet appeared to

offer some degree of protection against the hypercholesterolemic and hyperlipoproteinemic effects of the high saturated fat diet.

Partially purified triglyceride lipase activities from post-heparin plasma of both intact and supradiaphragmatic rabbit preparations were characterized using triglyceride emulsions as substrate. The preparations from intact rabbits contained both the hepatic and peripheral forms of triglyceride lipase. Selective measurement of these activities was carried out due to the almost complete inhibition of the peripheral lipase in the presence of protamine sulfate. The supradiaphragmatic enzyme preparation contained only the peripheral form of this lipase activity.

When radio-labeled VLDL from experimentally-fed animals (using semi-synthetic diets high in either saturated or unsaturated fat) were used as substrate for the above mentioned enzyme preparations, similar activities were observed whether preparations from intact or supradiaphragmatic rabbits were used. However, activities were lower than when normal chow-fed rabbit VLDL was assayed indicating that the VLDL from experimentally-fed animals is not as effective a substrate for the lipase enzyme. As a result, a build-up of the VLDL has been hypothesized under those dietary conditions which may be atherogenic. This finding is also consistent with the particle size of the various VLDL as estimated by surface to core ratios, the larger particles (i.e. chow-fed rabbit VLDL) being catabolized more rapidly.

The selective measurement of hepatic and peripheral triglyceride lipases from post-heparin plasma enzyme preparations of intact rabbits indicated a change in the relative contribution of the two lipase activities among the dietary groups. A relative decrease in the contribution of the peripheral lipase in the experimental diet-fed rabbit was observed. This decrease may

be important in that a build-up of altered VLDL particles in the circulation is occurring, accounting in part for the atherogenicity of the experimental diets.

The preparation of VLDL remnants from VLDL of rabbits fed the various diets was carried out in order to provide further information about the catabolism of both the altered and normal lipoprotein particles. The remnants prepared from chow-fed rabbit VLDL were triglyceride-poor and cholesteryl ester-enriched. Loss of phospholipid and apo C peptides occurred while the free cholesterol content remained about the same. In addition, the mobility of these remnants on agarose was, for the most part, beta, while the VLDL originally showed pre-beta mobility.

This result suggested that the VLDL isolated from rabbits fed either the high saturated or high polyunsaturated fat diets may be remnants of normal triglyceride-rich VLDL. However, the apolipoprotein content of this altered VLDL is such that it may be a unique lipoprotein derived via synthetic processes in the liver. Further work is necessary before the origin of this altered VLDL particle can be accurately determined.

The VLDL from either SAFF-fed or COCO-fed rabbits was already appreciably cholesteryl ester-enriched and triglyceride-poor. Yet when remnants were prepared using either of these VLDL, it became further triglyceride-depleted and cholesteryl ester-enriched. Surface to core ratios of these VLDL remnants were different, indicating that the COCO diet-fed rabbit VLDL remnant was a larger particle than that in the SAFF case. Although both VLDL lost about 40% of their respective triglyceride content, the additional amount of cholesteryl ester in the case of the COCO diet VLDL remnants may account for this larger particle. The size of these particles may be

related to their accumulation in the circulation in the event that their metabolism and utilization is somehow slowed or prevented.

Finally, the fatty acid composition of the VLDL was, in general, found to reflect that of the diets fed. The fatty acid composition of VLDL remnants, however, showed preliminary evidence for the preferential hydrolysis of linoleic acid by the triglyceride lipase preparations when VLDL is the substrate.

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